637.1:658

382. THE SEASONAL DISTRIBUTION OF CALF AND MILK SALES IN NORTH WALES AND THE PROBABLE INFLUENCE OF CLIMATIC CONDITIONS ON THE MONTHLY RATE OF CALVING AND CONSEQUENT MILK PRODUCTION

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(With 3 Figures)

In a previous paper two of the authors(1) reported that there is in the west Wales region a gradual cline from east to west in the distribution of autumn calf sales and in the seasonality of milk production. It is consequently of interest to determine whether or not similar features hold for the north Wales region consisting of the four counties of Anglesey, Caernarvon, Denbigh and Flint.

The data available for this study are drawn from the same sources as for those utilized for the west Wales region, namely (i) the sale of surplus calves through the collecting centres of the Ministry of Food; (ii) the sale of milk into the factories, calculated from the Milk Marketing Board data; and (iii) the agricultural returns of the Ministry of Agriculture and Fisheries. (The calf figures apply to the 4 years, 1943–6, but it has been necessary to confine the milk sales data to the 3 years, 1944–6.)

In *The Agriculture of Wales*, Ashby & Evans(2) have reviewed the agriculture for the four counties up to 1939, and have shown that whereas milk selling had long been established in Flint and in parts of Denbighshire it made rapid progress also in the more westerly counties of Anglesey and Caernarvon since the establishment of the Milk Marketing Board in 1933. The trend towards dairy farming was maintained during the war years and there appear to be ample opportunities for its continuation.

For reasons similar to those given for west Wales in the earlier paper (Phillips & Davies(1)) the study of north Wales has been confined mainly to the 4 years 1943–6, by which time the interaction of the wartime agricultural policy is considered to have become reasonably well defined.

As a result of the development of dairy farming, male calves in particular become surplus and are sold to the Ministry of Food. Consequently, the seasonality of the calf sales is a reflexion of the seasonality of the calvings.

SEASONAL DISTRIBUTION OF CALF SALES

(*a*) *Per county.* On a county basis the monthly sales as a percentage of the gross total sales have been calculated and are shown in Table 1. The county boundaries here are not necessarily the same as the administrative ones but are determined by the parishes allocated to the collecting centres and occasionally supplies to a particular centre are drawn from more than one county.

The monthly percentage figures show that Anglesey and Caernarvon on the one hand

and Denbigh and Flint on the other are similar in their trends. Denbigh and Flint show a high spring peak, occurring in March, and only a minor peak in the autumn, occurring in November in Denbigh and in October in Flint. By contrast, Anglesey and Caernarvon have only a moderate spring peak, occurring in March, but have a remarkably high autumn peak reaching its maximum in November in both cases. Denbigh and Flint have their lowest sales in June and July respectively while in Anglesey and Caernarvon the lowest points are in May and June.

Table 1. *Monthly percentage sales of calves in the north Wales counties of Caernarvon, Anglesey, Denbigh and Flint for the* 4 *years* 1943–6, *together with total calf sales during the period*

Counties	Jan.	Feb.	Mar.	Apr.	May	June	July	Aug.	Sept.	Oct.	Nov.	Dec.	Total no. of calves
Caernarvon	8·37	6·86	9·30	5·87	3·00	2·53	4·31	6·75	10·62	13·52	18·02	10·81	28877
Anglesey	8·63	7·06	8·30	5·19	2·32	2·45	3·95	7·77	13·40	14·81	16·65	9·57	13807
Denbigh	7·47	9·86	14·82	9·31	7·54	5·57	5·06	6·98	7·65	9·41	10·07	6·23	62507
Flint	7·43	8·70	12·71	7·41	6·19	4·74	5·69	8·46	10·03	11·39	10·29	6·83	26279

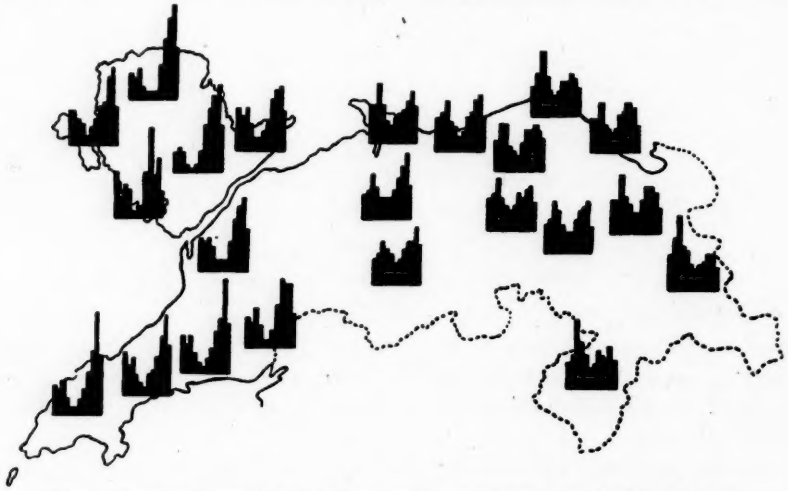
In order to express the differences between the two pairs of counties more clearly the figures in 4-monthly periods are shown in Table 2.

Table 2. *Calf sale percentages per* 4*-monthly periods*

County	Jan.–Apr.	May–Aug.	Sept.–Dec.
Caernarvon	30·40	16·62	52·98
Anglesey	29·18	16·39	54·43
Denbigh	41·46	25·18	33·36
Flint	36·25	25·21	38·54

The table shows that in the two westerly counties of Anglesey and Caernarvon there are considerably higher proportions of calf sales during the autumn months than in the more easterly counties of Denbigh and Flint. The division into two main groups is evident in the 4 months September to December, and this feature of the decline from west to east is in agreement with, although less marked than, that found in west Wales.

(*b*) *Per collecting centre.* In order to illustrate the east to west cline in the monthly distribution of the calf sales, the following map and graphs have been prepared for each collecting centre so as to give the average monthly proportions for the years 1943–6.

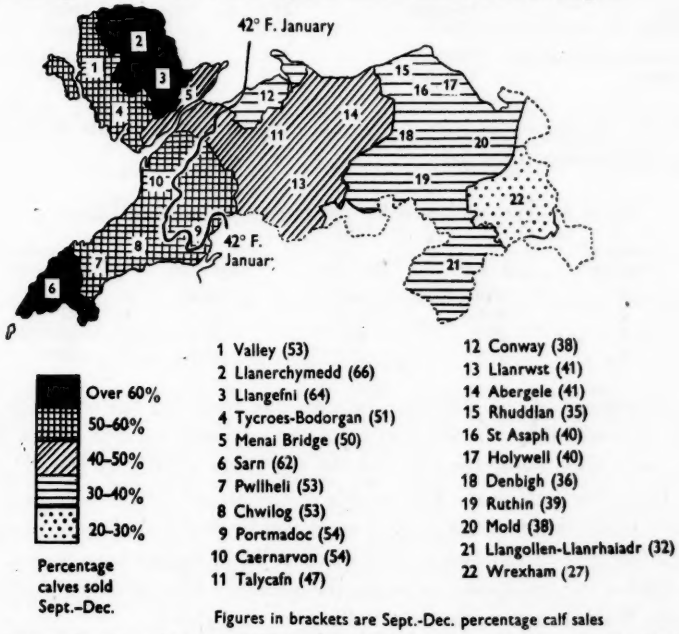


Map 1. Percentage monthly calf sales at collecting centres in north Wales, 1943–46.

It is seen that the graphs gradually change in form from the western end of the Lleyn peninsula in Caernarvonshire to the inland areas of Denbigh and Flint. In the western coastal centres the autumn peak dominates the whole graph and the spring peak is of minor importance only. Towards the eastern direction the graph assumes a better balance between spring and autumn, until finally in the inland areas of east Denbigh the spring peak dominates the graph. The gradualness of the change in the shapes of the graphs from west to east is difficult to understand in terms of husbandry methods alone, although it must be acknowledged that husbandry influences may be pronounced in attempts to regulate the distribution of calvings. Although it is known that during the war considerable changes in the proportions of autumn calvings have been effected, yet in view of the inducement to produce winter milk it is difficult to understand why—unless a climatic factor is operating over the region as a whole—the westerly districts in the north Wales peninsula, as in the west Wales peninsula, have been more successful than their fellow-farmers situated farther to the east and farther inland. It is not without significance that the Conway centre, with 38·4 % of its calves sold during the 4 months September–December, is well below Caernarvon with 54·1 % of its sales during the same months of the year.

It must be admitted that the more easterly areas in both regions—Flint in the north and Carmarthen in the west—have been concerned with milk production for direct sale for a longer period of time than the more westerly sister counties, yet as will be shown later in respect of first-calving heifers, the wartime difference between the two extremes is even more pronounced.

This west to east trend makes it possible to group together centres with similar autumn percentages of calf sales, and in this way to effect a system of territorial groupings as was previously made for the west Wales region. This is illustrated in Map 2.



Map. 2. Percentage of calves sold in north Wales collecting centres in the four-months period September–December for four years 1943–46 with 42° F. January isotherm superimposed. The actual percentage figure is placed after the name of each centre.

(*c*) *Territorial groupings of centres.* It will be seen from Table 3 how well the centres fit into the territorial groups.

Table 3 shows the fundamental difference between the groups in which the western ones have over twice the percentage autumn calf sales as the inland eastern group. This is further illustrated in Table 4 which gives the average percentage figures for each group for the 4-monthly periods.

Table 3. *The 4-monthly percentage calf sales (1943–6) in territorial groupings together with the gross number of calves for each centre*

			4-monthly percentages			
Group	Centres	Location	Jan.–Apr.	May–Aug.	Sept.–Dec.	Total no. of calves
1	Llanerchymedd	Anglesey	20·3	13·8	64·9	2000
	Llangefni	Anglesey	18·0	18·3	63·7	1739
	Sarn	West Caernarvon	24·5	13·4	62·1	2373
2	Chwilog	Caernarvon	30·2	14·7	55·1	5341
	Caernarvon	Caernarvon	29·4	16·5	54·1	8223
	Portmadoc	Caernarvon	27·2	18·7	54·1	2864
	Pwllheli	Caernarvon	30·8	15·8	53·4	6734
	Valley	Anglesey	26·2	20·9	52·9	1786
	Tycroes Bodorgan	Anglesey	31·8	17·5	50·7	1203
	Menai Bridge	Anglesey	34·9	15·4	49·7	7079
3	Talycafn	Denbigh	31·8	21·6	46·6	2217
	Abergele	Denbigh	35·9	22·8	41·3	10165
	Llanrwst	Denbigh	32·0	26·9	41·1	5572
	Holywell	Flint	34·8	25·5	39·7	10997
	St Asaph	Flint	34·1	26·3	39·6	3678
	Ruthin	Denbigh	35·8	25·4	38·8	5349
	Conway	West Caernarvon	40·0	21·6	38·4	3342
	Mold	Flint	37·6	24·3	38·1	8891
	Denbigh	Denbigh	37·7	36·0	36·3	5127
	Rhuddlan	Flint	40·4	24·9	34·7	2713
4	Llangollen and Llanrhaidr	Denbigh	40·1	27·8	32·1	2694
	Wrexham	Denbigh	47·0	25·7	27·3	32383

Table 4 shows the cross-over from a high autumn percentage in the western group to a high spring percentage in the east.

Table 4. *Average 4-monthly percentages per group (1943–6)*

Groups of centres	Jan.–Apr.	May–Aug.	Sept.–Dec.	Sept.–Dec. for group 1 = 100
1	20·9	15·2	63·9	100
2	30·1	17·1	52·8	83
3	36·0	24·5	39·4	62
4	43·6	26·8	29·7	45

Evidence in support of the difference between the west and the east with particular reference to autumn calving is obtained from the quarterly returns of the Ministry of Agriculture made since December 1939, on the 4th of the months of March, June, September and December each year. The records of the in-calf heifers have been examined according to a modification of the method employed by Smith (3) in order to estimate the number of heifers that calve down in each quarter. These figures have been averaged for the 4 years 1943–6 and transposed to obtain the quarterly rates of conception* for each of the four north Wales counties: the details are given in Table 5.

* I.e. the quarterly rates of successful services in in-calf heifers.

Table 5. *Quarterly rates of conceptions of heifers as a percentage of the annual totals in the four north Wales counties* (1943–6 *combined*)

County	Dec.–Mar.	Mar.–June	June–Sept.	Sept.–Dec.
Anglesey	49·6	20·6	8·2	21·5
Caernarvon	54·3	21·5	8·4	15·8
Denbigh	30·6	14·2	25·2	30·0
Flint	32·0	15·0	23·2	29·8

The table shows that the two westerly counties of Anglesey and Caernarvon have a considerably higher conception rate in heifers during the winter period December to March than have Denbigh and Flint, and it will be seen that the counties again pair remarkably well. This difference between the pairs is in agreement with a similar difference between them in respect of autumn calf sales, and support the view that the distribution of calf sales is an index of the seasonal distribution of the calvings. Smith (3) has shown that during the war years there was over the whole of Great Britain an increase in the rate of autumn calving amongst heifers with first calf, but it is seen from Table 5 that the successful attainment of this aim varies enormously from county to county. It may be stated also that where there is a low rate of autumn calving amongst heifers there is a high rate of spring calvings in the herds.

EVIDENCE FROM MILK SALES

Evidence in confirmation of the suggestion that the calf sales data are an index of the rate of calving, and that there is an east to west cline in the north Wales region, is obtained from the available data of the Milk Marketing Board.

(*a*) The index of seasonality of milk sales obtained by expressing the December output of milk as a percentage of the June output shows differences between the north Wales counties (Table 6).

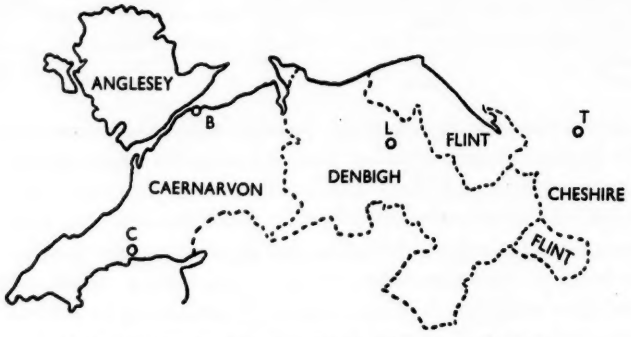
Table 6. *Index of seasonality calculated from the total sales of milk off farms* (*December as a percentage of June*) (M.M.B. *data*)

County	1941	1943	1945	1946	Average
Anglesey	86	84	92	104	91·5
Caernarvon	83	87	83	92	86
Denbigh	52	53	60	67	58
Flint	46	53	61	56	56·5

The data in Table 6 confirm the previous suggestion of differences between the two westerly counties of Anglesey and Caernarvon and the more easterly counties of Denbigh and Flint. The higher December milk sales in relation to the June sales of the two westerly counties support the evidence of the calf sales, inasmuch as a higher rate of autumn calving makes it possible to obtain a higher proportion of December milk. The converse regarding high rate of spring calvings is also true.

(*b*) *Deliveries of milk into milk factories and depots.* The location of the four milk factories in the four counties of north Wales is shown in Map 3. Although the Tarvin Factory is outside the boundary it draws considerable supplies from within the region. As it is not the purpose of this paper to deal with the total milk production of the county but only with its seasonal distribution it is of little consequence that some milk produced within the region is sold to factories outside the boundary of the four counties, provided that the factories obtain their supply from the same sources during the period under

review. The comparative figure obtained for each factory, or groups of factories as the case may be, should therefore be taken to show the main trends in milk production within the region.



Map. 3. Location of milk-collecting centres in north Wales.
B, Bangor; C, Chwilog; L, Llandyrnog; T, Tarvin.

The milk evidence is confined to 3 years 1944–6, and the monthly percentages have been calculated accordingly. As it is not permissible to publish details of milk deliveries into individual factories, it has been necessary to group the four factories in accordance with the similarity of their monthly percentages as follows: Group 1, Chwilog and Bangor depots (westerly group); Group 2, Llandyrnog and Tarvin depots (easterly group).

The 3-year averages for the monthly percentage of milk deliveries into the two groups of factories are given in Table 7.

Table 7. *Percentage milk deliveries into two groups of milk factories in north Wales* 1944–6

Group	Jan.	Feb.	Mar.	Apr.	May	June	July	Aug.	Sept.	Oct.	Nov.	Dec.	Total Sept.–Dec. (%)
1	8·5	8·1	8·9	8·7	10·5	9·7	8·1	7·0	6·3	7·3	7·7	9·1	30·4
2	5·4	5·5	7·1	8·5	11·5	11·6	11·0	10·0	8·7	8·1	6·4	6·2	29·4

The difference between the two groups in Table 7 is that which would be expected between winter milk producers and summer producers, and this bears out the evidence of the calf sales.

It is also of interest to note that in Table 7 the total percentage deliveries during the 4 months September to December (30·4% in Group 1 and 29·4% in Group 2) are very similar and practically coincide with the figures for the west Wales groups of milk factories in which the average was found to be 28·8%. This seems to suggest that whatever the rate of calving in an area the milk deliveries, and presumably the milk production during the autumn months September to December, is a fairly constant proportion of the annual production and is approximately 30% of that total.

Although the autumn milk percentages (September to December) are fairly constant proportions of the annual total in the above two groups of factories, yet its make-up is quite different as shown in Table 8, where the September percentages are taken as 100 in each case.

Table 8. *Comparison of the September–December percentages of milk deliveries into two groups of factories with September taken at* 100 *in each case*

Group	Sept.	Oct.	Nov.	Dec.
1	100	115·5	122·2	144·4
2	100	93·1	73·5	72·1

The western group of factories (Group 1) show an increasing rate from September to December, whereas the eastern group has a downward trend, and by December its proportion is only half that of Group 1.

PERCENTAGE DRY COWS

The quarterly returns of the Ministry of Agriculture also provide supporting evidence of the difference between the two pairs of counties which roughly approximates to the collecting areas of the two groups of factories.

The average percentage of the dairy and breeding herd (cows and heifers in milk, cows in calf and not in milk) in milk on each quarter day (4 March, 4 June, 4 September and 4 December) for the 4 years 1943–6 is given for each county in Table 9.

Table 9. *Cows in milk as a percentage of the total dairy and breeding herds during* 1943–6 (*quarterly returns of the Ministry*)

County	4 Mar.	4 June	4 Sept.	4 Dec.
Anglesey	75·6	86·3	78·7	75·5
Caernarvon	79·5	83·3	74·5	76·7
Denbigh	67·8	84·1	78·6	66·2
Flint	68·5	85·6	77·7	65·0

Although in the summer quarter days (4 June and 4 September) the percentage figures for all counties are approximately the same, there is a definite contrast between the two pairs of counties on the recording days during the winter (4 December and 4 March). It is evident that the general picture is in agreement with the calf and milk sales data already discussed.

It is of interest to note, however, that practically all the figures for the north Wales counties are at a higher level than those given for the counties in the west Wales region, and actually the lowest figures in the above table are equal to the highest for the west Wales peninsula. Whether this difference between the north and west reflects influences other than husbandry is difficult to say, but Roberts (4) has shown that in north Wales the Welsh Black breed which dominated the westerly counties of Anglesey and Caernarvon compares favourably with the Shorthorn breed, which in the main dominated the west Wales peninsula.

CLIMATIC CONDITIONS

The evidence afforded by the detailed analysis of calf sales is in accordance with that found in west Wales (Phillips & Davies (1)). It remains to be shown that the climate as a whole, or one or more elements which make up the climate, provide a satisfactory explanation of the decline in autumn calving in the period October–December from west to east.

The problem, as before, is to determine which climatic element is responsible for this cline and if more than one is involved how they interact to give the final picture. It has been suggested that the amount of bright sunshine in the period of conception December–March will materially affect the proportion of successful conceptions and therefore the proportion of autumn calvers. This is already supported by two lines of evidence (1) by Yeates (5) on the effect of light on season of reproduction in sheep; and (2) by the correlation between high autumn calving and high spring sunshine in west Wales. (1)

Map 2 shows the simplified picture of autumn calvings in north Wales. There is an overall decline from west to east. The decline is, however, not an even one, a point of some considerable significance, as will be shown later. It is suggested that this decline is due to

the contracted winter conditions in the western coastal areas, particularly in Anglesey and Lleyn compared with the late spring and early autumn of Denbigh and Flint.

Taking Holyhead in western Anglesey as representative of the western end of the line and Sealand (near Chester) of the eastern end, then Table 10, of mean monthly minimum temperatures, shows the temperature conditions at this vital period of the year.

Table 10. *Mean monthly minimum temperatures*

	Dec.	Jan.	Feb.	Mar.	Apr.	May
Holyhead	42·5	41·6	39·9	40·3	42·4	46·5
Sealand	37·1	36·9	35·8	35·5	39·0	43·5

If a minimum temperature of 42° F. is taken as being the lower limit of vegetative growth, then it is seen that Holyhead at the beginning of the year is at least 1 month (April) earlier than Sealand. At Holyhead the mean monthly minimum temperature is at no time less than 39·9° F., and grass growth is not seriously impeded for any length of time in mid-winter. At Sealand the critical months December–March are all well below 42° F., even April has a mean monthly minimum temperature of 39·0° F. which is below the lowest at Holyhead. It is not until May (43·5) that uninterrupted grass growth occurs.

The same considerations apply at the autumn end of the year; grass growth does not cease until January in the west, but in the east by November the mean monthly minimum has dropped to 38·2° F. and effective grass growth has ceased.

A consideration of the percentage of possible sunshine actually received at the two stations in the early months of the year shows that the western station, although enjoying little advantage in December and January, soon outstrips the eastern station from February onwards as Table 11 shows.

Table 11. *Percentage of possible bright sunshine actually received*

	Dec.	Jan.	Feb.	Mar.	Apr.	May
Holyhead	18	21	27	34	42	41
Sealand	17	21	22	32	34	43

By April the west receives 8% more than the east. This is equivalent to an average of over half an hour more per day.

In north Wales, as in west Wales, there is a high correlation between high percentage of autumn calvers and early spring sunshine, late and early grass growth.

It has been noted that the cline from west to east is not an even one, there are certain very obvious anomalies (see Map 2). In Anglesey the north-east (Llanerchymedd and Llangefni) has a greater percentage of autumn calvers than the south-west (Valley and Tycroes-Bodorgan). This is the reverse of what is expected. In the absence of any climatic data for the rest of Anglesey it is impossible to offer any explanation.

There remains an even greater anomaly on the main land—one that has been noted already in the paper. The Conway collecting area forms an enclave of 30–40% calves sold September–December within an area where a percentage of 40–50% prevails. The Conway area is more akin to the Rhuddlan, St Asaph, Denbigh areas than to its immediate neighbours Talycafn and Menai Bridge.

A consideration of the climatic data available for Aber (within the area) provides an explanation.

In Table 12 are tabulated the mean monthly minimum temperatures for Holyhead, Aber and Sealand for December–May.

Table 12. *Mean monthly minimum temperatures*

	Dec.	Jan.	Feb.	Mar.	Apr.	May
Holyhead	42·5	41·6	39·9	40·3	42·4	46·5
Aber	39·1	39·0	37·4	39·0	42·0	45·9
Sealand	37·1	36·9	35·8	35·5	39·0	43·5

Temperature conditions at Aber lie between those at Holyhead and Sealand. This is what is normally expected from a consideration of its location between the two extremes. But the calf data (Map 2) suggest that Conway is not intermediate between west and east but is similar to the area of which Sealand is representative.

When sunshine values are tabulated (Table 13) a very different picture emerges.

Table 13. *Percentage of possible sunshine actually received*

	Dec.	Jan.	Feb.	Mar.	Apr.	May
Holyhead	18	21	27	34	42	41
Aber	7	13	22	34	34	37
Sealand	17	21	22	32	34	33

Although the percentages differ widely, Aber is more like Sealand than Holyhead. During February to March the two are almost identical and in December and January Aber is actually less than Sealand.

The anomaly of a low percentage of autumn calvers in Conway coincides with a low percentage of December–March sunshine in that area. The temperature conditions seem to have no direct bearing on the problem in that minimum temperatures in Conway are normal for its location. The very low December and January sunshine in Aber may account for the major part of the low proportion of autumn calvers.

Having shown that the amount of sunshine in the December–March period is apparently correlated with the percentage of autumn calvers from west to east, and also for the variations found within the general picture, it remains to consider how temperature exerts its influence, if at all.

If, as seems to be the case, the amount of bright sunshine exerts such a big influence, then those areas where the herds are exposed to the direct sunlight at this period (December–March) will be more favourably placed for a high percentage of autumn calvers than those areas where the herds are habitually housed at this time of the year. But the necessity for housing depends largely upon exposure and upon the amount of grass available which is in turn dependent upon the duration of the temperature below 42° F. In those areas where grass growth is but little checked then the herds will be out most of the year, certainly earlier than those where a longer period below 42° F. is the rule. Thus exposure to sunshine in the critical early months also depends indirectly upon temperature conditions, the warmer the conditions the earlier the grass growth and the greater exposure to sunshine.

DISCUSSION

It has been shown that the most favoured areas with a high minimum temperature and a high percentage of bright sunshine in the December–March period are the western coastal margins, and so in these particularly favoured areas the direct amount of sunshine and indirectly the temperature combine to give the best conditions for autumn calving.

There is a reduction of sunshine and minimum temperatures from west to east—the fall not being an even one. This overall decline and the variations within the decline are

faithfully reflected in the decline of autumn calvings from west to east. There is no doubt that husbandry methods influence the results, but the general and gradual cline from west to east in the calf records suggests that the climatic influence is the most important. The contention that the extent of arable cropping shows a high correlation with winter milk production in so far as it influences the quantity and quality of the winter foods available for winter feeding of the dairy stock, is not borne out in the north Wales counties since the tillage area as a percentage of the total crops and grass is very similar at 33–38% for all four counties. Caernarvonshire, which shows the highest rate of autumn calvings and produces the highest proportion of winter milk, has actually, during the years under review, the lowest percentage (33%) of tillage to total crops and grass. The suggestion that the nutritive value of the fodders may vary materially within the region is probably correct as it is known that weather conditions at harvest have a profound effect on their quality.

SUMMARY

There appears to be a west to east cline in the four north Wales counties in: (i) the proportion of calves sold during the autumn months September to December; (ii) the proportion of December milk sales in relation to the September sales; (iii) the proportion of the dairy and breeding herd in milk during the winter; (iv) the quarterly rates of conception in heifers during the period 4 December to 4 March. There does not appear to be any relation between percentage winter milk production and percentage tillage area in this region. It is postulated that the west to east cline is dependent mainly upon factors other than husbandry and that climatic conditions, particularly the hours of bright sunshine during the winter months, have a marked influence upon the breeding performance of cows and heifers during the period December to March.

This investigation has formed part of the survey of animal health and production in Wales, and thanks are due to the scientific branch of Messrs Cooper, McDougall and Robertson, Berkhamsted, for financial support. Grateful acknowledgement is made to the Ministry of Food, Colwyn Bay, for readiness in providing data and helpful advice; to Messrs Pritchard, Auctioneers, Bangor, and Messrs Beardmore, Auctioneers, Abergele, for permission to examine their records; to the Milk Marketing Board for providing the data on milk sales; to Profs. A. N. Worden and E. G. Bowen for the interest they have taken in the work.

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383. INVESTIGATIONS ON BOVINE MASTITIS

4. THE ASSOCIATION OF NEW INFECTIONS CAUSED BY *STREPTOCOCCUS AGALACTIAE* WITH (*a*) SEASON AND (*b*) STAGE OF LACTATION

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For some time before special investigations on bovine mastitis were begun at this centre, the observation was repeatedly made that milk samples submitted for the diagnosis of this disease appeared to reach maximum numbers for the year in the course of the late summer or early autumn. While a proportion of such cases was attributable to *Corynebacterium pyogenes* a high percentage of positive samples contained *Streptococcus agalactiae*. In later years, as the study of the disease progressed and data accumulated, the apparent association of agalactiae mastitis with late summer and autumn was frequently observed, and the desirability of obtaining a sufficient volume of data on the subject was one of the major considerations influencing the decision to continue the work throughout a considerable number of years. The extension of the investigation to include the influence of lactation suggested itself at a later date when, in the course of the working up of preliminary data on the effect of season, it became obvious that allowance would require to be made for dates of calving.

METHODS

The herds which provided data for these studies are given in Table 1.

Table 1. *To show particulars of herds*

Herd	Licence	Self-contained (S) or partially flying (F)	No. of byres	Average no. of cows	Tested	
					From	To
A	Certified	S	2	30–65	Spring 1939	Winter 1946
B	Certified	S	2	40–70	Spring 1939	Winter 1946
K	Standard	S	2	40	Spring 1939	Summer 1942
L	Standard	At first F	2	40–50	Spring 1939	Spring 1945
M	Certified	S	3–4	90–140	Spring 1939	Winter 1946
O	T.T.	S	2	40–60	Spring 1939	Spring 1946
P	Certified	S	2	50–70	Spring 1939	Winter 1946
R	Standard	At first F, S from 1946	1	40	Autumn 1939 Winter 1944	Spring 1942 Winter 1946
U	Certified	S	1	45	Winter 1939	Spring 1942
V	Standard	F	1	70–90	Winter 1940	Spring 1945
Ga	T.T.	S	3	55	Spring 1945	Winter 1946
Ha	T.T.	S	1	40	Winter 1945	Winter 1946
Ka	T.T.	S	1	40	Winter 1945	Winter 1946
Qa	T.T.	S	1	40	Winter 1945	Winter 1946

S = recruited from home-bred heifers only.
F = partially dependent on purchases.

The herds selected were tested at approximately 3-monthly intervals and the annual results were recorded in four columns headed spring (February to April), summer (May to July), autumn (August to October) and winter (November to January). For the study of the influence of stage of lactation the data were re-classified into five groups, four 3-monthly stages, and a fifth stage for lactations which had not terminated at the end of the previous

* The death of Dr Cunningham occurred on 4 March 1949, when this paper was in press.

four. The lactation stages began nominally at the date of parturition, but in the case of cows as distinct from heifers, the first stage generally included a dry period and frequently a small part of the previous lactation. This was due to the fact that the animals were tested only at 3-monthly intervals. A few herds were tested for a short period only but in all cases only completed years and lactations were utilized. Infections which occurred in 1939 were omitted because in many of the herds testing only began in that year.

The methods of sampling and bacteriological examination were those described in *Tech. Bull. Edinb. Coll. Agric.* nos. 1 and 2(1), (2). In the critical consideration of the results only data which satisfied certain criteria of new infection were included. In order to obtain some assurance that infections were of recent date no record was accepted unless the animal had been tested at least twice previously with negative results or was a heifer. Records which showed that an animal had missed more than two quarterly tests immediately preceding infection owing to the occurrence of a dry period or for some other reason were also omitted. In period *a* (1939–42) the standard of infection demanded the presence of at least twenty colonies per plate, or alternatively presence of a smaller number associated with clinical evidence, or with *Str. agalactiae* in other quarters, or with confirmatory evidence obtained in subsequent tests. In period *c* (1944–6) all new infections were confirmed with reference both to cow and quarter by a re-test carried out within 3 weeks of the first. In both periods no cow which had previously shown infection attributable to streptococci other than *Str. agalactiae* was included; such animals are liable to harbour small numbers of *Str. agalactiae*, the presence of which may be concealed by overgrowth of the other organisms and may thus cause uncertainty in determining the date of the new infection. Previous infection with staphylococci was not regarded as a sufficient reason for omitting an agalactiae infection because material from such cases was replated on crystal violet agar in order to ensure that it did not contain *Str. agalactiae*. The data for the years 1942–4 (period *b*), during which enrichment methods of diagnosis were employed, were only included in cases in which they had been confirmed. Experience in long-term testing indicates that if the results of enrichment tests are confirmed very few false positives are recorded.

RESULTS

(*a*) *Association of new infections with season*

Some indications of a seasonal variation in percentage of new infections with a maximum number occurring late in the year can be observed in certain of the graphs reproduced in *Tech. Bull.* no. 2(2). Such results can be found in fig. 2, especially in 1940, 1944 and 1945, and in fig. 5, especially in 1941, 1943 and 1944. It must be observed, however, that these are little more than mere indications and that in many years the maxima occurred earlier. It should be recalled that the graphs shown in *Tech. Bull.* no. 2(2) are based on uncorrected results and that some of these were not even confirmed. The data were obtained for purposes of eradication of infection caused by *Str. agalactiae*, and no cow which had once given a positive result was again recorded as negative, although confirmation of infection may have been unobtainable. For the purposes of this paper it is inadvisable, therefore, that entire reliance should be placed on these results. A rough assessment of the demonstrated association of new infection with season, obtained by recording for each herd the season in each year (1940–6) in which the maximum number of new infections occurred, gave the following figures:

Spring	Summer	Autumn	Winter
8	10	13	6

The distribution of the figures suggests that the maximum occurs in early autumn. The total uncorrected figures for new infections recorded in the graphs were:

Spring	Summer	Autumn	Winter
87	101	103	57

They are in fair agreement with the maxima.

The first step in the preliminary elaboration of the data was the application of the criteria for valid new infections detailed in the introduction to this paper. As a consequence of these restrictions the number of new infections was reduced from 348 to 212. The corrected figures for new infections were then tabulated in seasons by years and herds.

Table 2. *To show new infection by years* (1940–6)

Maximum figures are shown in heavy type

Year	Spring	Summer	Autumn	Winter
1940	16	3	16	9
1941	11	**15**	13	5
1942	8	5	**12**	6
1943	6	6	**10**	3
1944	3	**7**	6	4
1945	4	5	**10**	7
1946	6	4	**8**	4
Totals	54	45	75	38
Maxima	0	2	4	0

Table 2 shows the numbers of new infections arranged by years under the seasons. It may be noted that in 1940 spring and autumn figures are equal and are maximal values, in 1941 the maximum is in summer, while in 1942 to 1946 inclusive the maxima are, with one unimportant exception, in the autumn periods. The data also indicate that the maxima are substantially higher than the 25% demanded by the assumption of equal frequencies and in all cases equal or exceed one-third of the new infections for the year. The majority of the yearly maxima occur in the autumn period.

Table 3. *To show new infection by herds* (1940–6)

Maximum figures are shown in heavy type

Herd	Spring	Summer	Autumn	Winter
A	**3**	0	1	0
B	**7**	2	**7**	6
K	1	1	**3**	2
L	2	**8**	5	3
M	**17**	11	15	9
O	3	3	**7**	0
P	8	8	**18**	12
R	2	3	3	0
U	**4**	2	**6**	0
V	**10**	6	4	4
Ga	1	0	**3**	1
Ha	0	0	**1**	0
Ka	0	0	**1**	0
Qa	0	1	1	1
Totals	54	45	75	38
Maxima	3	1	7	0

The numbers of new infections arranged by herds under the seasons are set out in Table 3. In this tabulation seven of the eleven recorded maxima fall within the autumn period. In herds A, M and V, however, the maxima are in spring, while in herd L the maximum is in summer. Reasons for the abnormal behaviour of herds L, M and V will be discussed later.

The aggregate corrected new infections by seasons for the years 1940–6 are as follows:

Spring	Summer	Autumn	Winter
54	45	75	38

It is clear that the maximum occurs in the autumn period.

Up to this point there is a fair measure of agreement among the corrected results. The most notable discrepancies occur in 1940 and 1941, the first two years for which valid figures are available, and in herd M. In the case of this herd the seasonal distribution of new infections shows no resemblance to the average for all herds. The spring maximum and the greater uniformity in the seasonal results distinguish the data from those for the majority of the others. Throughout most of the investigation herd M showed a consistently high level of new infections following injuries. If injury is more potent than season as a factor predisposing to infection the abnormally high proportion of injuries occurring in this herd might account for the unusual seasonal distribution.

The exceptional characteristics of the data for 1940 and 1941 seem to indicate that in those years factors may have been in operation which no longer exerted an influence in the later stages of the investigation. Careful consideration of possible causes of the irregularities suggested that they may have been associated with the partially flying herds tested during that period. It is obvious that frequent introduction of infected cows from other herds must cause considerable disturbances in the infection status of the herds concerned. The reservoir of infection tends to be maintained or increased, but probably a still more serious factor is the continuous introduction of new strains of the organism. There is great risk that some of the latter may spread with exceptional rapidity among cows which have not previously been exposed to them. This is in fact what actually appears to have happened in at least two of the herds of the type mentioned, in which some of the most active and acute infections encountered during the whole investigation were recorded. Evidence of this may be found in the graphs for herds L and R (*Tech. Bull.* no. 2(2)), both of which showed a very high incidence of new infection in the first half of 1941. In the majority of the self-contained herds, on the other hand, infection was less active; it was generally of the chronic, slowly spreading type. It is reasonable to anticipate that the two types of the disease will behave differently not only in regard to the rate at which they produce new infections but also to the characteristics of the latter, and that such differences will be intensified by the conditions prevailing in the flying herds. It is considered, therefore, to be advisable to exclude the new infection data for the partially flying herds at least for the period during which considerable numbers of cows were purchased from outside sources. This involves the exclusion of the new infections recorded in herds L, R and V during the years 1940–1. The alteration reduces the new infection data to 176 distributed in the following manner:

Spring	Summer	Autumn	Winter
45	31	67	33

The revised herd maxima are distributed as follows:

Spring	Summer	Autumn	Winter
3	0	8	0

When these distributions are compared with those shown in Table 3 it is obvious that the flying herds must have played a part in maintaining the relatively high values recorded in the earlier seasons of 1940 and 1941.

Under certain circumstances an obvious explanation of the characteristic seasonal distribution of new infections may be found in an association of calving dates with a particular season, coupled with the occurrence of maximum new infections at a certain stage of the lactation. For example, if the majority of the infections occur in the first stage of the lactation the characteristic seasonal distribution of new infections observed in this investigation can be accounted for if the majority of the calving dates occur in early summer. If, however, the distribution of new infections noted throughout most of this investigation is reproduced when the cows are selected, so that their calving dates occur in equal numbers in each of the seasons, the evidence will support the view that the distribution is in fact solely or mainly attributable to season and not to an appreciable degree to the combined effects of calving date and stage of lactation. In order to eliminate the influence of lactational factors cows were selected at random in groups of four, so that one calving date fell within each of the seasonal periods. The seasonal distribution of calving dates of the cows included in the data corrected for criteria of valid new infection, flying herds being excluded, is as follows:

Spring	Summer	Autumn	Winter
56	39	42	39

The method of random selection adopted reduces still further the volume of available figures. New infections are reduced from 176 to 156 and their distribution becomes:

Spring	Summer	Autumn	Winter
39	27	60	31

The figures are the means of six distinct random selections. They show a clearly defined autumn maximum, the figure 60 being significantly different from the values for spring, summer and winter. This is confirmed when new infections unselected in groups of four (see Table reproduced on p. 142) are divided by the numbers of calvings occurring in the respective seasonal periods. The values then become:

	Spring	Summer	Autumn	Winter
	0·80	0·79	1·60	0·85
Or approx.	1	1	2	1

(*b*) *Association of new infections with stage of lactation*

The data employed for this part of the investigation were the corrected figures already used under season; the flying herds were again eliminated. When the corrected new infections were distributed in the appropriate lactation stages the following result was obtained:

Lactation stage	1	2	3	4	5
New infections	69	50	37	17	3

The figures show a progressive decline as the lactation proceeds.

It is essential to eliminate the possibility of an influence of season upon the results. For this purpose the data were subjected to random selection to ensure that the seasonal periods were equally represented among the new infection dates. When this was done the lactation figures were found to be:

Lactation stage	1	2	3	4	5
New infections	49	32	28	13	2

The figures are the means of six distinct random selections. The progressive decline, therefore, appears to be confirmed.

The lactation data are also subject to a special type of limitation not previously referred to. This arises from the fact that many cows fail to continue to milk for a normal lactation period. A consequence is that the lactation data tend to be much more numerous for the early than for the late stages of the period. The figures can be corrected by calculating the new infection values for each stage of the lactation as percentages of the numbers of previously uninfected cows tested during that time. Owing to lack of calving dates for certain herds the calculation of numbers of infections per lactation stage had to be restricted to three large herds for which accurate records were available. These herds contained in all from 200 to 300 cows during the whole period of the investigation, a number believed to be sufficiently representative for the purpose. The percentage new infections calculated as described are as follows:

Lactation stage	1	2	3	4	5
% new infections	3·96	3·17	2·82	3·23	5·00
S.E.	±0·57	±0·53	±0·57	±0·88	

Omitting the fifth period in which the numbers were too small to yield an accurate result it is obvious that, although more infections are recorded in the first than in any of the subsequent lactation stages, the differences are not statistically significant. There is no evidence of a uniform trend in the figures.

DISCUSSION

As already stated the data discussed in this paper were not collected with the primary object of securing evidence on the association of new infection with season and stage of lactation. They were obtained in the course of an attempt to ascertain whether infection attributable to *Str. agalactiae* could be controlled or eliminated by segregation and disposal of infected animals. The association of the recorded dates of new infections with certain seasons was observed at an early stage of this investigation, and during its further progress results bearing on the subject were collected. It is recognized, therefore, that the plan of the work is not ideally suited to the object in view. Some of the precautions taken to check, and if possible to increase, the reliability of the results have already been described. With few exceptions the modifications introduced as checks have made little alteration in the general trend of the results, a fact which tends to emphasize the significance of the data. In this section it is proposed to discuss a number of additional factors which may influence the reliability of the conclusions to be drawn and to attempt to evaluate their importance.

The period of 3 months between tests may appear to be unduly prolonged and to intensify the risk that certain new infections may be missed entirely. It should, however, be stated that available records of the results of continued testing of recently infected cows show that about 65% persist in a detectable form for periods exceeding 3 months, and therefore may be expected to be diagnosed by the methods employed.

A consequence of failure to detect new infections would be that many re-infections of cows which had become agalactiae-negative might be mistaken for new infections. Considerable numbers of re-infections have been recorded in the course of this investigation, and a study of the more detailed of such records serves to throw some light on the question of the risk of failure to detect new infections. Thus the distribution of the dates of twenty-two well-authenticated re-infections in the seasons in which they occurred yielded the following result:

Spring	Summer	Autumn	Winter
	7	5	2

In this case the maximum occurs early in the year and the distribution is quite distinct from that which has been found to be characteristic of new infections in this investigation. Although the data are restricted the observations tend to support the view that the infections recorded in the foregoing sections of this paper are in fact new and not mere re-infections.

It may appear that two negative tests immediately before infection do not provide a sufficient assurance that the animal concerned has not been infected at an earlier date. The results of repeated testing of infected cows are known to be subject to marked fluctuations. It should be emphasized, however, that two negative records was the minimum number accepted. Many cows which ultimately became infected had been tested repeatedly with negative results. Details are shown in Table 4. The numbers of negative pre-infection tests for 154 cows ranged from 2 to 24; averages per cow ranged from 4·2 to 8·2 in the periods *a* to *c*. In addition, 21 first-lactation animals were found to be infected in the first and second tests.

Table 4. *To show the numbers of pre-infection negative tests*

		Pre-infection negative tests			
			Tests per cow		Heifers infected in first and second tests
Period	Cows tested	No. of tests	Average	Range	
(*a*)	45	188	4·2	2–11	7
(*b*)	68	533	7·8	2–16	8
(*c*)	41	338	8·2	2–24	6

There is no reason to doubt the ability of the bacteriological methods employed to diagnose correctly the new infections. The methods have been thoroughly tested for a variety of purposes. Their reliability has been assessed and found to be satisfactory. In the work described three distinct techniques were employed at different times(1). Table 5 shows the results obtained by the different methods. In each division of the table the individual maxima all occur in the same columns which also include the maximum of the total figures. This suggests that the methods may be considered to have been adequate for the purpose for which they were used.

Table 5. *To show details of seasonal new infection data*

Season...	Spring	Summer	Autumn	Winter
Period				
(*a*)	18	4	**21**	9
(*b*)	17	18	**28**	13
(*c*)	10	9	**18**	11
Totals	45	31	**67**	33

The elimination of the flying herds was one of the alterations which produced a very definite increase in the uniformity of the data by causing the figures for 1940 and 1941 to approach more closely to those for subsequent years. The seasonal distribution of the new infections omitted in consequence of the elimination of the flying herds shows that these results bear no resemblance to the data regarded as characteristic of the remainder of the work. The figures omitted were:

Spring	Summer	Autumn	Winter
9	14	8	5

The maximum is in the first half of the year.

It seemed reasonable to anticipate that the elimination of influences, due to the stage of the lactation, from the data on season would enhance the reliability of the results. The evidence already presented shows clearly that the observed seasonal and lactational effects are quite distinct and not interdependent. It appears permissible, therefore, to conclude that the distributions recorded in preceding pages are in fact attributable to seasonal and lactational influences respectively and not to their interaction.

It is evident that as the tests carried out in each season or stage of the lactation were spread over a period of 3 months, and as approximately 3 months elapsed between tests, new infections recorded in a particular season or stage of lactation may have occurred at any time within the 3–6 months preceding the date of testing. It is, therefore, clearly impossible to determine with a high degree of accuracy the date on which a particular infection occurred. In relation to maximum infection, however, an attempt has been made to define more precisely the periods to which the results apply by re-classifying the data by the months in which they were recorded. The results are shown in Table 6.

It should be noted that the results of tests carried out in successive months refer to different groups of herds and are not strictly comparable. The influence of a particularly active group of herds is seen in the months January, April, July and October. This group generally included the herd M with which was associated an unusual seasonal distribution. This fact may account for the relatively high new infection values recorded in January and April. The autumn maximum is discernible in the monthly results and is seen to include the months August–October. The period is clearly defined by the relatively low values which occur before (May, June and July) and after it (November and December).

Table 6. *To show the months in which new infections were recorded*

	Infections recorded in											
Calendar month ...	Jan.	Feb.	Mar.	Apr.	May	June	July	Aug.	Sept.	Oct.	Nov.	Dec.
No. of infections	19	6	15	24	9	7	15	24	14	29	5	9

A further limiting factor associated with the dry period may be operative in the seasonal data for certain animals. The arbitrary division of the infection data into groups corresponding to periods of 3 months fails to take adequate account of the dry period. As a rule the first test after calving covers an interval which includes that during which the animal was dry as well as a small portion of the previous lactation. It is, therefore, impossible to determine from data of the type quoted in this paper whether the infection has occurred in the early stages of the current lactation, or in the previous dry period or in the late lactation before the animal became dry. For the purpose of distinguishing between the early current lactation and the previous dry period or late lactation the examination of colostrum just before parturition has much to commend it.

It is evident that for heifers infected in the first stage of the first lactation the factor associated with the dry period is inoperative. Comparatively few animals become infected at this stage. The fact that the first calving is not preceded by a dry period may be a factor in producing this result.

In the case of a cow one or more 3-monthly tests may fall outside the period of lactation. This means an interval of 6, 9 or more months may elapse between the last test in one lactation and the first test in the next. A new infection detected in the first test after calving could have arisen at any time during that period.

It is possible to obtain some evidence regarding the reliability of the seasonal data as they are affected by the occurrence of dry periods from a careful study of the results. Of the selected new infections recorded in Table 3:

(*a*) 65·9% of the animals were in milk at the test immediately preceding infection.

(*b*) 16·5% missed one test immediately preceding infection.

(*c*) 5·7% missed two tests immediately preceding infection.

(*d*) 11·9% were heifers infected in the first or second test.

It is obvious therefore that 22·2% of the new infections were preceded by one or more periods in which no tests could be made. Such results can be eliminated from the seasonal data without appreciably impairing the reliability of the remaining figures. When this is done the selected seasonal data (p. 142) become:

Spring	Summer	Autumn	Winter
32	23	54	28

It is clear, therefore, that tests missed during the dry period have exercised little influence on the occurrence or magnitude of the autumn maximum.

On the question of the association of new infection with season and stage of lactation little or no evidence has been found in the literature which deals with bovine mastitis. Detailed records of tests of individual cows extending over considerable periods are quoted by Seelemann (3), but unfortunately the majority do not refer to new infections. In sixteen cases, however, clear evidence of new infection is presented and the distributions of the infection dates are as follows:

Season	...	Spring	Summer	Autumn	Winter
		3	3	7	3
Stage of lactation	...	1	2	3	4
		5	6	3	2

Although very limited in volume the results are in close agreement with the figures quoted in this investigation.

The remaining records of the association of the disease with season and stage of lactation do not refer specifically to new infection. Many of these investigations deal with clinical mastitis. Seasonal associations are referred to by Stylianopoulos (4) and by Arthur (5). The latter found that clinical mastitis (the aggregate of all types) reached a maximum for the year in August and September.

On the association of the disease with stage of lactation Klein & Learmonth (6), Kästli (7), Stylianopoulos (4) and Arthur (5) have made contributions. The data, however, show little general agreement, although the last-named three workers noted a high incidence of infection in late lactation or in the dry period.

SUMMARY

Regular testing at 3-monthly intervals for periods extending to 7 years of some 650 to 850 cows in fourteen herds indicated that the majority of the new infections caused by *Str. agalactiae* were recorded in the autumn months, August, September and October.

When infections were considered in relation to the stage of lactation, although higher new infection values were recorded at the first test after calving than at later stages, the differences were not statistically significant.

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384. A NOTE ON THE EFFECT OF PENICILLIN IN THE REDUCTASE TEST FOR MILK QUALITY

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The methylene blue or reductase test is used in New Zealand as a means of estimating the bacteriological quality both of milk for liquid consumption and of milk for cheese-making. The minimum reduction time standards are different for the two classes of milk. Regulations governing the quality of milk intended for liquid consumption require that the raw milk as delivered to pasteurizing plants, or to consumers, shall not decolorize methylene blue in less than 4 hr. The reductase test on milk for cheese-making is carried out in conjunction with a rennet curd test, and milk decolorizing the blue in less than 2 hr., or giving an unsatisfactory curd test, suffers a price penalty.

The reductase test result is usually taken as an index not only of the keeping quality of the milk, but also of the sanitary conditions in the milking shed, although it is well known that the cooling of milk on the farm limits the effectiveness of the test as a criterion of milking machine sanitation. Residual penicillin in milk from cows given the penicillin treatment for masitis has been found to retard the growth of cheese-starter cultures (1, 2). Following on this finding, it seemed possible also that these residual quantitites may delay the growth of contaminant bacteria in milk samples, and thus introduce one more confusing factor into the relationship between sanitary conditions on the farm and the reductase time of the milk produced. It was considered of interest, therefore, to investigate the effect of penicillin in milk on the results of the reductase test.

METHODS

I. Composite samples of night's and morning's milk were collected each day on delivery at the Institute factory. Control samples, and 10 ml. quantities of the different milks to which penicillin was added in concentrations ranging from 0·10 to 10·0 units/ml., were submitted to the methylene blue test immediately. A duplicate set of samples, with and without added penicillin, was held at room temperature (63–65° F.) for 24 hr. and was then examined by the reductase test. The technique suggested by Wilson (3) with half-hourly inversion of the tubes was adopted throughout.

II. Penicillin in concentrations of 0·10, 1·0 and 2·0 units/ml. was added to portions of the milk samples in sterile flasks. Three sets of 10 ml. quantities from each of the flasks and appropriate controls were then transferred to test-tubes, and held at 41, 72 and 86° F. respectively for 16 hr., after which time they were adjusted to a temperature of 98–99° F. Methylene blue was then added and the times taken for complete reduction of the colour were noted.

RESULTS

The following points emerge from a survey of the results:

I (*a*) *Milks submitted to the reductase test immediately after the addition of penicillin*

(1) The time taken for the control samples (no penicillin added) to decolorize the blue varied from 2 hr. to more than 8 hr.

(2) The samples containing penicillin took from 2 to 5 hr. longer to decolorize the blue. In some of the poorer grade samples, this difference meant that a milk passed the legal 4 hr. test, where in the absence of penicillin it would not have done.

(3) Penicillin concentrations of 0·10 and 0·50 unit/ml. were generally as effective as were the higher concentrations up to 10 units/ml. Only occasionally in this series of tests did the time vary significantly with increasing concentrations of penicillin.

I (*b*) *Milks treated with penicillin and submitted to the reductase test after standing 24 hr. at room temperature*

(1) Control samples after standing 24 hr. decolorized the blue in 10 min. to 2 hr.

(2) The samples containing penicillin took from 0 to 2 hr. longer than their appropriate controls to decolorize the blue. In the majority of milks the difference was less than $\frac{3}{4}$ hr. 1·0 penicillin unit/ml. and over prolonged the reductase time in some milks to reach a 2 hr. standard and in an odd sample to a 4 hr. standard.

II. *Milk samples held at* 41, 72 *and* 86° F.

(1) The samples, with and without penicillin, after standing at 41° F. for 16 hr., were all of good quality and did not decolorize methylene blue in less than 8 hr.

(2) After being maintained at a temperature of 72° F. for 16 hr., the control samples showed a reduction time of 1–7 hr. The time taken by samples containing 0·10 penicillin unit/ml. varied from $1\frac{1}{4}$ hr. to more than 8 hr., and those containing 1·0 and 2·0 units/ml. from 2 hr. to more than 8 hr.

(3) The control samples held at 86° F. and then submitted to the test reduced the blue in $\frac{1}{2}$–$4\frac{1}{4}$ hr.; the samples containing penicillin reduced the blue in from $\frac{3}{4}$ hr. to more than 8 hr. at the 0·10 unit/ml. level and from 2 hr. to more than 8 hr. at the 1·0 and 2·0 units/ml. level.

(4) In this series of tests, the larger amounts of penicillin frequently had much more effect in prolonging the reduction time than the smaller amounts, and consequently, while the lower concentrations in the incubated samples increased the time from 0 to 2 hr., the higher concentrations increased it from $1\frac{1}{2}$ to over 6 hr.

The results as a whole indicate that penicillin in milk has a definite but variable effect in prolonging the reductase time. The variability of the effect is not surprising when the mixed bacterial population of penicillin sensitive and non-sensitive types is considered. Of the two main contaminant types of bacteria in milk, viz. the streptococci and coliform organisms, the lactic streptococci are sensitive to penicillin and the coliform bacteria non-sensitive. Hence variation in the relative proportions of these two types alone will exert a marked effect on the result. It is also in accord with expectations that penicillin prolongs the reductase time more markedly in a fresh milk than in an aged milk. Any factor which stimulates or restrains bacterial growth has a more evident relative effect when the culture is in the lag phase than it has when the culture is already in the process of rapidly increasing the reduction potential.

Thus the presence of penicillin in milk as a result of the treatment of cows for mastitis may sometimes give an improvement in the quality of milk, as judged by the reductase test, delivered to a depot or cheese factory. This improvement is likely to be more marked in fresh morning's milk than in evening's milk delivered next morning, and more marked in well-cooled milk than in uncooled milk. The improvement in quality is in a sense artificial because it does not indicate a corresponding improvement in sanitary conditions or in

efficiency of cooling on the farm. To that extent it is misleading just as an apparent improvement in quality due to the presence of a preservative like formalin would be misleading. Thus the possible presence of penicillin in milk and its effect in the reductase test make the quality results more difficult to interpret. In dealing with problems which may arise in connexion with milk quality it is as well for the investigator to be aware of the influence of penicillin.

SUMMARY

Penicillin in milk inhibits the growth of some contaminant organisms and thus may have a variable effect in the reductase test for milk quality.

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385. GROWTH REQUIREMENTS OF LACTIC STREPTOCOCCI. DIFFERENCES WITHIN THE GROUP

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(With 2 Figures)

Some time ago we obtained, through the courtesy of Mr L. J. Meanwell, United Dairies Ltd., England, a streptococcal culture for use as a cheese starter. This culture (ML) was maintained in daily subculture in milk together with our other stock strains of lactis and cremoris, and it showed no peculiarities until subjected to the usual plating and purifying procedure on lactose-yeast-phosphate-agar (1). Streaked on the surface of this particular medium, culture ML failed to grow within 48 hr. Furthermore, later experience with ML as a cheese-starter showed that it varied more than our other cultures in its acid-producing power in various milk supplies at different periods of the dairying season. These indications of special requirements for active growth of ML organisms were investigated with the following results.

1. CHARACTERISTICS OF CULTURE ML

The culture grew readily in milk at 22 and 30° C., clotting the milk in about 18 hr. and reducing the pH to 4·25–4·30 after 24 hr. Incubated in milk at 37° C. for 4–6 hr. it showed chains of varying length, the cocci being somewhat involuted. It grew readily at 10° C. but was completely inhibited at 40° C. The culture was of medium vitality compared with other strains in use as starters for cheese-making. In other routine differentiation tests, it behaved similarly to stock strains of *Streptococcus cremoris* (Orla-Jensen), except that in fermentation tests it was able to utilize maltose (Table 1). Pentoses, trisaccharides, alcohols, polysaccharides including dextrin, and the disaccharide saccharose were not fermented. Sherman & Hussong (2), Sherman (3) have described substrains of *Str. cremoris* varying in their ability to attack maltose. Orla-Jensen (4) states that *Str. cremoris* grows feebly, or hardly at all, in maltose and never in dextrin. Thus the general physiological properties do not permit a clear-cut differentiation of type, but the culture has been tentatively classified as a variant of *Str. cremoris*.

Table 1. *Fermentation reactions of ML. Acidity in parts per thousand as lactic acid*

Basal medium: Sugar-free casein-digest broth containing 0·5% N.

Levulose	Glucose	Mannose	Galactose	Maltose	Lactose
4·9	4·4	3·5	4·0	2·7	4·5

Growth on solid media

When streaked on the surface of the stock lactose-yeast-phosphate-agar medium, containing B.D.H. peptone the culture did not give colonies within 48 hr. at 22° C., although some growth did occur after 4–7 days' incubation. It was known (1) that with most strains of *Str. cremoris* the brand of peptone used in the medium has a marked effect

on the size of colony produced in 24 and 48 hr. A trial of ML with different peptones gave the results shown in Table 2 and furnished further evidence of the need for specific nitrogenous materials in the medium in order to ensure rapid growth of individual strains of streptococci. Results obtained with a stock strain of *Str. cremoris* (HP) are included in the table for comparison.

Table 2. *Growth of strains ML and HP on solid media containing different peptones*

Medium: agar 2%, beef extract 1%, lactose 2%, peptone 1%, yeast extract 10%, $Na_2HPO_4.2H_2O$ 0·6%, pH 6·8. Incubation temperature 22° C.

	Growth; average colony size, diameter in mm.					
	1 day		2 days		4 days	
Peptone	ML	HP	ML	HP	ML	HP
Bacto-peptone Difco	—	±	—	++0·40	—	++0·45
Peptone B.D.H.	—	+++0·25	—	++++0·85	+0·10	++++0·90
Proteose-peptone, Difco	—	+++0·25	—	++++0·9	++0·40	++++0·90
Neopeptone-Difco	—	++++0·55	±0·18	++++1·0	+++0·55	++++1·0
Tryptose-Difco	—	+++0·20	+0·20	++++0·9	+++0·60	++++0·9
Tryptone	+0·20	+++0·25	+++0·60	++++1·0	++++0·70	++++1·0

Tryptone gave the most rapid growth of ML and the largest colonies in 48 hr. Tryptose and neopeptone were next in order, although the growth was relatively poor. B.D.H. peptone which was used successfully in routine work with other organisms failed to stimulate growth of ML within the standard incubation period of 2 days.

Trials with media containing varying amounts of tryptone and B.D.H. peptone showed that the growth of ML was in proportion to the amount of tryptone present in the mixture (Table 3), and that the poor response on the stock medium was due to the lack of stimulating substances in the B.D.H. peptone rather than to the presence of inhibiting materials. A 1% peptone mixture comprising tryptone 0·12%, B.D.H peptone 0·88%, was required to initiate growth within 48 hr.; a 4+ growth was obtained in 48 hr. when equal quantities of the two peptones were present, and all concentrations of tryptone above 0·12% in the mixture gave 4+ with large colonies in 72 hr. No growth was obtained on the control B.D.H. peptone medium within that time.

Table 3. *Effect of addition of tryptone to stock medium containing* 1% *B.D.H. peptone*

Strain ML. Incubation temperature 22° C.

	Growth; average colony size, diameter in mm.		
	24 hr.	48 hr.	72 hr.
Stock medium control	—	—	—
+1% tryptone	+0·16	++++0·67	++++0·72
+0·4% tryptone	±0·10	++++0·45	++++0·70
+0·2% tryptone	?	+0·16	+++0·53
+0·1% tryptone	—	±0·11	++0·40
+0·05% tryptone	—	?	+0·30

The substitution of B.D.H. peptone by tryptone in the solid agar medium ensured an adequate supply of suitable food materials and permitted the early production of 'normal' colonies from which pure and active substrains could be obtained. Thus it was possible to include culture ML in the normal routine of regular replating, which is followed in this laboratory with all single strain cultures used for cheese-making. As detailed below, further

experience showed the culture to be relatively fastidious as compared with other stock strains of *Str. cremoris* and *Str. lactis*.

(1) *The effect of free oxygen*

Comparison of growth of strain ML with other stock strains of lactic streptococci in stab culture, agar slope with heavy butt, and agar shake cultures prepared from the standard medium containing B.D.H. peptone, made it clear that in such a medium culture ML would grow, but only under semi-anaerobic conditions. This effect is shown diagrammatically in Fig. 1, and the preference is further illustrated in Table 4 which gives details of results obtained when strains ML and HP were streaked on the surface of different media and incubated aerobically and under conditions of reduced oxygen tension.

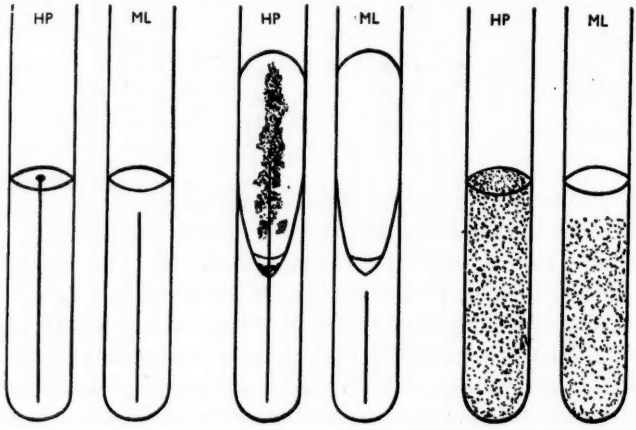

Fig. 1. Growth of strains HP and ML on lactose-yeast-phosphate-agar containing B.D.H. peptone. Temperature 22° C. Time 48 hr.

Table 4. *Growth of strains ML and HP in relation to reduced oxygen tension*

Media: I. Agar medium containing lactose, B.D.H. peptone, beef extract, yeast extract, phosphate.
II. Agar medium containing lactose, tryptone, beef extract, yeast extract, phosphate.
III. Agar medium containing glucose, B.D.H. peptone, beef extract, yeast extract, phosphate.
pH 6·8. Temperature of incubation 22° C. Time 48 hr.

	Growth; average colony size, diameter in mm.					
	Aerobic Medium			Semi-anaerobiosis Medium		
Strain	I	II	III	I	II	III
HP	++++0·99	++++0·80	++++0·54	++++0·94	++++0·84	++++0·83
ML	Nil	++++0·72	++++0·43	+++0·36	++++0·80	++++0·53

The results indicate that the replacement of lactose by glucose in the medium stimulates rapid growth of ML under aerobic conditions even when B.D.H. peptone is the main nitrogen source; and further that ML is able to use lactose in a medium containing B.D.H. peptone only when the oxygen tension is reduced. Tryptone, on the other hand, stimulates growth and utilization of lactose even in the presence of free oxygen.

It is also of interest to record that the American Public Health Association standard agar for milk analysis (5) gave good growth of ML colonies averaging 0·50 mm. in diameter

within 48 hr., whereas no growth occurred with the British Milk standard agar (Wilson (6)), presumably because the former medium contains tryptone and glucose, and the latter B.D.H. peptone and lactose.

(2) *Effect of added materials to the stock agar medium*

Tryptone is a peptone recommended by Digestive Ferments Company as especially suitable for indol production by bacteria because it is rich in tryptophane. The addition of tryptophane to the original stock medium (based on B.D.H. peptone), however, failed to stimulate growth of ML. Skim-milk additions up to 5% in the final medium did not stimulate growth, but hydrolysed casein (5%) gave a + growth with small colonies averaging 0·20 mm. in diameter in 48 hr., and 4+ with colonies 0·55 mm. in diameter in 72 hr.

The observation that growth of ML was dependent in some media upon a low oxygen tension suggested that the addition of strongly reducing substances, for example, sodium thioglycollate, to the stock medium might provide a more suitable oxidation-reduction potential. This substance proved to be non-toxic but ineffective in concentration of 0·01–0·10%, although other compounds, for example, cystine, cysteine and glutathione containing the sulphydryl group, produced some stimulant action (Table 5). Other reducing materials, for example, ascorbic acid (neutralized) and succinate, 0·1% concentration, gave no growth in 48 hr., but 4+ (0·30 mm.) and 3+ (0·20 mm.) respectively in 72 hr.

Table 5. *Effect of addition of cysteine, cystine and glutathione to stock medium containing B.D.H. peptone* pH 6·8

Strain ML. Incubation temperature 22° C.

	Growth; average colony size, diameter in mm.		
	24 hr.	48 hr.	72 hr.
Stock medium control	—	—	—
+0·25% cysteine, partly insoluble	±0·11	+ + + +0·37	+ + + +0·45
0·10%	?	+0·15	+ + + +0·44
0·01%	—	±0·08	+ + +0·35
0·001%	—	±0·09	+ + +0·30
0·0001%	—	?	+ +0·23
+0·10% cystine, partly insoluble	?	+ +0·24	+ + + +0·50
0·05%	?	+0·14	+ + + +0·33
0·01%	?	+0·20	+ + +0·44
+0·25% glutathione	?	+ +0·23	+ + + +0·53
0·10%	?	+0·09	+ + + +0·38
0·01%	—	±0·08	+ +0·21
0·001%	—	±0·09	+0·23
0·0001%	—	±0·06	+0·21

All the evidence at this stage of the investigation suggested that the value of tryptone lay in the fact that some part of it was an accessory factor capable of stimulating early colony growth of culture ML, or that it contained reducing compounds which were able to establish a redox potential more favourable to the utilization of lactose in the medium. Measurement of the initial potential with the gold electrode showed no significant difference between media containing tryptone or B.D.H. peptone. At pH 6·8, temperature 22° C., E_h values ranging from +0·11 to +0·15 V. were obtained, with individual electrodes in agreement within 5–10 mV. The complexity of the systems present in the medium, some of which may not be readily or completely reversible, make it difficult, however, to

ascertain with certainty that the problem is one of oxidation-reduction potential. On the other hand, without recourse to purely synthetic media, it is not possible to decide whether or not the early stimulation of growth is due to the presence in tryptone of a vitamin-like substance or to specific groupings. The nitroprusside test for —SH groups was negative on both peptones and on media prepared from them.

(3) *The effect of* pH

During the trials, in which different materials were added to the agar, it was noted that ML would grow more rapidly on the original stock medium when the pH was relatively low. Table 6 gives details of results obtained on streaking ML organisms on agar (containing B.D.H. peptone), the pH of which was adjusted (*a*) by addition of the appropriate amounts of phosphate solution, and (*b*) by addition of HCl to the full medium containing phosphate. The time or stage of adjustment, i.e. before or after sterilization, appeared to have little effect.

Comparisons were also made of growth of ML and other lactic streptococci on both B.D.H. peptone and the tryptone media adjusted to pH levels ranging from 7·6 to 6·0, and it was again evident that culture ML is more sensitive to variation in pH than are other strains of *Str. cremoris*. Culture HP, for example, showed measurable growth in 48 hr. at pH 7·3 on both types of medium, while the upper limit for growth of culture ML on tryptone agar was 6·9. It would grow on B.D.H. peptone agar only when the pH was below 6·7, maximum colony formation being obtained at 6·4. In all cases the lower pH levels were characterized by a reduced clotting power of the colonies picked into milk.

Table 6. *Growth of strain ML on stock medium at different* pH *levels*

Temperature 22° C.

pH	Growth; average diameter of colony in mm. 24 hr.	48 hr	72 hr.
	(*a*) Adjustment of pH with phosphate solution		
6·8	—	—	—
6·7	—	?	+ + +0·20
6·4	?	+ + + +0·27	+ + + +0·30
6·0	?	+ + + +0·23	+ + + +0·26
	(*b*) Adjustment of pH with HCl		
6·8	—	—	—
6·7	—	+0·17	+ + + +0·38
6·6	—	+ + + +0·31	+ + + +0·38
6·4	±	+ + + +0·37	+ + + +0·40
6·2	±	+ + + +0·32	+ + + +0·35
6·1	±	+ + + +0·31	+ + + +0·35
6·0	?	+ + + +0·10	+ + + +0·19

It is evident from the foregoing data that culture ML is more exacting in its growth requirements than other strains of lactic streptococci so far investigated in this laboratory. For rapid growth on the surface of a solid medium it seems to require the presence of some specific nitrogenous compound, or at least some special chemical grouping. Tryptone supplies this unknown factor, whereas the other brands of peptone tested contain either a mere trace or none at all. The required factor for growth cannot be supplied by non-specific reducing agents.

When the factor is absent from the medium, culture ML can grow rapidly only under conditions of semi-anaerobiosis or of low pH. This seems to indicate that the culture uses a different metabolic mechanism according to the composition of the medium. The fact

that a change in pH of the 'deficient' medium enables the culture once more to grow aerobically is interesting. Topley & Wilson (7) quote a similar phenomenon with *Corynebacterium acnes*. The effect of low pH in the medium without tryptone is to delay growth, not to prevent growth entirely, and once the organism has produced enough acid round a small colony the lowering of the pH then helps growth under aerobic conditions.

From a practical point of view the results obtained with tryptone as compared with other peptones have important implications. For many years in this laboratory a search has been made for active strains of lactic streptococci for use as cheese starters. It is now evident that strains similar to ML, if present in any material examined, will have been missed entirely, although they may have been active acid formers in liquid milk. Is it not possible that still further types whose requirements for growth on the surface of a solid medium are not satisfied, even by the tryptone medium, are still being missed? The observation recorded above emphasizes the false picture of a bacterial population which may be obtained by a plating procedure.

2. GROWTH IN MILK

When culture ML first came under observation, any significant differences in behaviour from that of other stock 'starter' streptococci were made evident only when comparisons were carried out on solid media. The organisms appeared to grow normally in milk and were of average activity as regards acid production. Later, however, it became obvious that any abnormality in milk composition influenced the growth of ML more readily than that of other cultures.

Acid-producing activity of the culture

The activity of a starter culture is of great importance in cheese-making, and the vitality test technique (8) furnishes a method for determining the relative rates of acid production of different strains of lactic streptococci under conditions somewhat similar to those obtaining in the cheese vat. Tests were carried out frequently over an entire season. The results are summarized in Fig. 2. Monthly averages of acidity figures at the first and second titrations are given. A comparison of strain ML with an active strain HP is shown, and it is clear that the latter remained remarkably constant in activity over the whole season. ML, on the other hand, was relatively active over the first 5 months, but fell away in the following 2 months during which a drought was experienced in the district from which the milk was drawn. At the end of the dry spell, the activity increased. During the short period when the milk was 'abnormal' it was found that additions of tryptone to the vitality-test milk gave a marked increase in acid production (Table 7). Several tests were made and tryptone regularly gave a significant increase in acid production, whereas the effect with B.D.H. peptone was relatively small. With the more active culture HP, both tryptone and B.D.H. peptone gave only a slight increase in the acidity figures.

The large increases brought about by the tryptone additions were only apparent during the short period the milk was (presumably) abnormal. Other factors which influenced the growth of ML on solid media did not appear to play a similar role in liquid milk. Any differences shown by culture ML as compared with other stock strains under similar conditions were not constant. Colonies of ML picked from an agar medium into milk gave a fair percentage of slow clotting cultures which were characterized by a failure to coagulate

milk except in the lower half of the tube. Attempts to select fast and slow clotters by subculture from the top and bottom layers of the clotted tubes (9) did not yield substrains of significantly different vitality.

Table 7. *Addition of tryptone to vitality-test milk*

Strain ML as starter. Acidity as parts per thousand lactic acid

Test milk	1st titration figure	2nd titration figure	Increase
Control	2·1	3·9	1·8
+0·2% Tryptone	2·5	6·2	3·7
+0·1% Tryptone	2·7	6·3	3·6
+0·05% Tryptone	2·8	5·9	3·1
+0·01% Tryptone	2·3	5·2	2·9

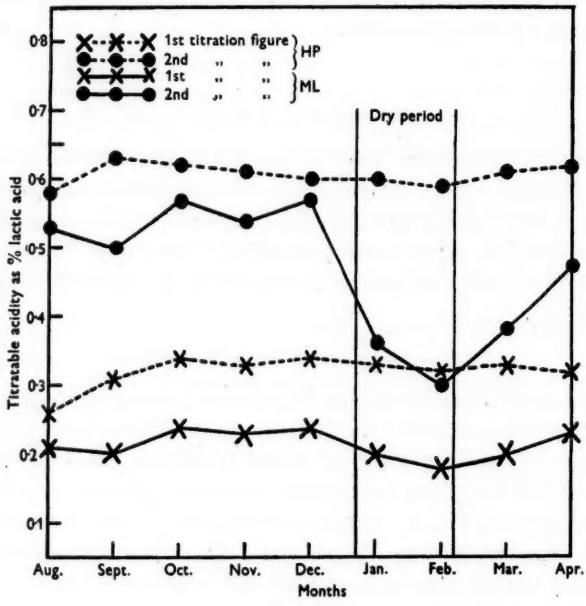

Fig. 2.

Behaviour of culture ML in commercial practice

During the summer months, attention was drawn to variation in behaviour of the culture in different factories. Compared with other starters, it showed a more marked 'slowness' in some districts where pastures were dry. It was not possible to make a survey of all factories, but results obtained in two adjoining districts are of interest. Over the dry period when pastures showed little or no fresh growth or were very dry the cheese milk at the Institute factory assumed an 'abnormal' character in that it was low in initial acidity and coincidently acid development in the vat when ML was used as starter became considerably slower. With improvement of the pastures following rain the initial acidity at 'setting' rose from 0·155 to 0·17% and a more normal performance of the starter was experienced. Changes in acid production were noticeable to the cheese-maker the day

after rain, and a clear-cut difference was apparent on succeeding days. It was possible to reduce the amount of starter used by ¼ to ½% and still complete the manufacturing process 30–35 min. earlier than in the drought period prior to rain. Thus at the Institute the behaviour of ML in the cheese milk ran parallel, as might be expected, with its behaviour in the vitality test. The culture was quite distinctly more sensitive than other single strains of *Str. cremoris* to changes in the cheese milk. There was no sudden change in the gross chemical composition of the milk corresponding to the change in behaviour of ML culture with the advent of rain after the drought period.

Trials in which 0·01% of tryptone was added to the cheese milk were only possible after the pastures were recovering. At the beginning additions of tryptone gave an improvement in acid production up to 'drying' time. The experimental vat 'dried' out 10 min. earlier than the control at the same acidity. The cheeses were firmer in body at maturity corresponding to the better acid production in the vat. When the milk returned to 'normal' as evidenced by a higher 'cutting' acidity the addition of tryptone gave no improvement over the controls.

In contrast to these findings, the performance of the same culture in a neighbouring district was constant throughout the summer months. Three parts of the total cheese milk were drawn from swamp land which was not greatly affected by the dry spell, the remaining one part was from hilly country. The only difference noticeable to the cheese-maker was the need to increase the 'ripening' period 5 min. in order to obtain comparable 'drying' acidities within the same time. Immediately after the dry spell the 'ripening' was reduced 5 min. and the normal constant acid production was obtained.

Unfortunately, there was no opportunity at the time for further direct proof of milk differences or abnormality, but it is apparent that culture ML is affected by changes in milk composition. In the past many references have been made by various workers to the possibility of some milks being less suitable for growth of starter organisms owing to a deficiency or even absence of specific growth factors. In New Zealand milk differences have been postulated as a cause of variation in starter behaviour, in different districts, but definite evidence for this has so far been lacking. In this instance, the more fastidious ML culture has shown response to milk changes which were not reflected by the behaviour of more hardy lactic streptococci.

Up to the present there is no direct correlation between the behaviour of an organism in liquid and solid media. The differences in growth characteristics which may be exhibited by strains of lactic streptococci are amply illustrated by the results described. Strain ML has proved very useful as a cheese starter in normal milk; yet it is much more particular as regards the composition of a solid medium. It seems likely that different metabolic mechanisms are involved under different conditions. Thus, although a plating procedure is employed for isolation and purification of starter cultures, it need not necessarily give a true indication of the value of a particular organism as an acid producer in the cheese vat. Probably the rapid acid-producing cultures so far obtained in New Zealand have been isolated only because they possess metabolic activities which can be readily adapted to a wide range of artificial conditions for growth.

SUMMARY

A variety of lactic streptococcus which is unusual in its growth requirements is described. The culture grows on an agar medium (based on B.D.H. peptone) only under conditions of semi-anaerobiosis. It will grow on the surface, however, if the medium is altered in certain respects, for example: (*a*) if the B.D.H. peptone is replaced by tryptone, the only peptone which appears to stimulate rapid aerobic growth of the organism; or (*b*) the lactose in the medium is replaced by glucose; or (*c*) the pH of the medium is reduced.

Different metabolic mechanisms appear to be involved according to the composition of the medium.

The culture is more sensitive to changes in milk composition than are other strains of lactic streptococci.

The author is indebted to Dr H. R. Whitehead for helpful criticism.

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386. VARIATIONS IN MORPHOLOGY OF *STREPTOCOCCUS LACTIS* WHEN GROWN IN RAW AND HEATED MILK

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(With 2 Figures)

INTRODUCTION

During the course of investigations arising from the possibility of the production of heat-resistant reducing substances in raw milk by *Streptococcus lactis* (1), it was found necessary to carry out Breed counts when the inoculated milk had been kept at atmospheric temperature until a reading of disk 0 was reached in 10 min. on the resazurin test, in order to estimate the approximate number of organisms present so that appropriate dilutions could be made to estimate the number of viable organisms by the colony count test. Microscopical examination of the Breed smears prepared disclosed that some organisms grew in long chains and not in the form of diplococci or short chains, as generally described in recognized text-books. Smears made for microscopical examination of the above-mentioned organisms when grown in litmus milk showed them to be morphologically typical strains of *Str. lactis* as the organisms were only present in the form of short chains and diplococci.

The only apparent reason for this difference in morphology was that the organisms were inoculated into 'sterile' raw milk during the experiment, whereas the litmus milk had been sterilized by intermittent steam sterilization. A series of experiments was then planned to find whether there was a constant variation in morphology when *Str. lactis* was grown in raw and heated milk.

EXPERIMENTAL

Cultures of lactic streptococci were isolated from raw milk samples of poor bacteriological quality, and those conforming to *Str. lactis* on Hunter's (2) classification were used during the following experiments.

It is of interest to note that 17% of cultures isolated in this way were found to be *Str. lactis* var. *maltigenes* which is a slightly higher percentage than recently reported by Nichols & Hoyle (3), suggesting that these variants are more prevalent than generally supposed.

Variations in morphology of different cultures when grown in raw and heated milk

In order to prove whether there was a constant variation in morphology when the organisms were grown in raw and heated milk, the following technique was adopted.

The numbers of organisms present in 18 hr. old litmus milk cultures were estimated by carrying out Breed counts on suitable dilutions made in sterile physiological saline. An inoculation from each culture of approximately 2,000,000 organisms per ml. was then made into tubes containing 10 ml. of 'sterile' raw milk and tubes containing 10 ml. of

milk that had been heated to 70° C. for 1 hr., these tubes were then incubated in a water-bath at 37–38° C. for 6 hr., Breed smears being made from each tube at hourly intervals, and the difference in morphology of the organisms recorded.

The cultures examined in this way were classified into three main groups as follows:

(1) Organisms growing in long chains in raw milk and short chains and diplococci in heated milk.

(2) Organisms growing in clumps in raw milk and short chains and diplococci in heated milk.

(3) Organisms growing in short chains and diplococci in both raw and heated milk, but the average length of chain was invariably longer in raw milk than in heated milk.

In the case of the strains of *Str. lactis* producing a malty taint in milk, no correlation could be found between length of chain and the formation of the taint.

Effect of variations in length of chain formation on the colony-count test

Previous investigations by Morris (4) confirmed the report by Hobbs (5) that there appeared to be a growth inhibitory factor present in raw milk responsible for very much lower colony counts on raw milk than on milk heated to 70° C. for 1 hr. when inoculated with pure cultures of different organisms and incubated at 37° C. for 6 hr. We found (6) in experiments carried out, using pure cultures of *Str. lactis*, that the colony counts on inoculated raw milk were much lower than on inoculated heated milk, but the time for complete reduction of methylene blue was very similar. As there is a close agreement between the rate of reduction of methylene blue and the number of organisms present, when the organisms are recognized as quick reducing types, it was considered that this lack of correlation might be due to *Str. lactis* producing short chains in heated milk, and in consequence forming a large number of colonies, and long chains in raw milk, and so forming a smaller number of colonies, although the actual number of individual cells was the same.

To prove whether there were the same number of individual cells present in raw and heated milk, a method had to be devised whereby a total count of the individual cells could be made. This could only be accomplished microscopically, and for this purpose Breed's method was modified in the following way. The milk smears were prepared and stained according to the technique described by Wilson (7) and an ordinary Breed count carried out to estimate the number of chains present. Then the average number of cocci in 50 chains, picked at random, were estimated and the total microscopical count (T.M.C.) calculated by multiplying the Breed count by the average number of cocci in a chain.

Experiment adopted to find the influence of length of chain on the colony-count test

As previously described, *Str. lactis* cultures were inoculated into raw milk and milk heated to 70° C. for 1 hr. to give an approximate inoculum of 2,000,000 organisms per ml., and the following tests carried out during a 6 hr. period of incubation in a water-bath at 37° C.: (1) colony count test at hourly intervals; (2) T.M.C. at hourly intervals; (3) a methylene blue test at the time of inoculation.

It was found impossible to obtain an accurate T.M.C. at the time of inoculation and for the first hour after incubation because many of the organisms were only feebly stained, but

after 2 hr. incubation this difficulty was not encountered. The results of these tests for some of the cultures examined are summarized in Table 1 and seem to support the theory that the low colony counts on the raw milk are closely correlated to length of chain formed, and the rate of proliferation of the organisms is approximately equal in both raw and heated milk, which would account for the methylene-blue reduction times being similar.

Table 1. *Comparison of colony counts,* T.M.C., *at* 4 *hr. and methylene-blue complete reduction times of* Str. lactis *in raw and heated milk*

Culture no.	Colony count at inoculation	Colony count after 4 hr. at 37° C.	Average length of chain after 4 hr. at 37° C.	T.M.C. after 4 hr. at 37° C.	Methylene-blue reduction time (hr.)
(1) Raw	2,790,000	4,900,000	21·25	46,601,000	4
Heated	2,660,000	56,400,000	1·7	36,218,000	4
(2) Raw	610,000	40,200,000	2·5	63,133,000	4½
Heated	620,000	86,000,000	1·4	67,470,000	4½
(3) Raw	960,000	12,300,000	6·9	38,439,000	5½
Heated	910,000	50,600,000	1·0	38,500,000	5½
(4) Raw	900,000	26,800,000	3·6	91,340,000	5½
Heated	780,000	86,500,000	1·0	91,884,000	5½
(5) Raw	2,440,000	11,700,000	11·0	144,000,000	4
Heated	2,800,000	91,400,000	1·75	176,000,000	4
(6) Raw	1,150,000	74,000,000	1·0	62,244,000	4
Heated	1,120,000	88,000,000	1·2	79,583,000	4

The effect of length of chain on the colony-count test during the 6 hr. incubation can be shown better in the form of a graph. (See figures 1*a* and 1*b*.)

Comparison in raw and heated milk of T.M.C. *and the colony-count test of a* Str. lactis *culture forming long chains in raw milk and* Str. lactis *culture forming short chains in raw milk*

It was found impracticable to carry out any T.M.C. on the raw milk when an organism that formed clumps was under test, as after 3 hr. incubation the clumps became so dense that it was impossible to estimate the number of individual cocci present, and for this reason no correlation could be attempted between colony counts and T.M.C. in raw and heated milk for clumping organisms. However, colony counts were carried out at hourly intervals on inoculated raw and heated milk during incubation, and similar results were obtained to those shown above for long chain-forming organisms. This similarity between the long chain-forming organisms and the clumping organisms suggested that the lower colony counts in the raw milk were not caused by an agglutinating factor as there was no decrease in colony counts during the maximum period of proliferation, although microscopical examination of Breed smears showed that large clumps were forming. To verify this assumption it had to be proved that the organisms were not agglutinated, that is, drawn together to form clumps, but that each organism grew and formed a clump, and this could only be shown by direct microscopical observation during the incubation period. Milk could not be used for this experiment as the presence of fat globules made it impossible to see the organisms clearly, therefore whey was used after first proving that the organisms formed clumps in whey the same as in milk. Hanging-drop preparations of whey inoculated with young, actively growing clumping strains to give an approximate inoculation of five or six organisms per field were prepared and placed on a warm stage controlled at 37° C. and the organisms continually observed until large clumps were

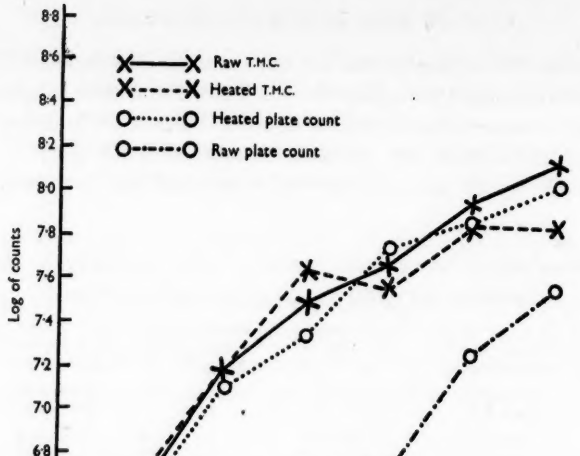

Fig 1*a*. Comparison of colony counts and total microscopical counts in raw and heated milk of a strain of *Str. lactis* producing long chains in raw milk.

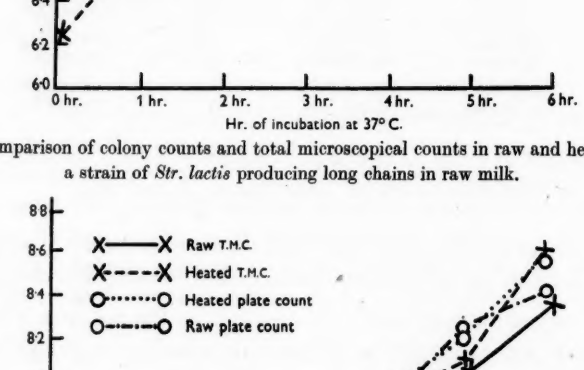

Fig. 1*b*. Comparison of colony counts and total microscopical counts in raw and heated milk of a strain of *Str. lactis* producing short chains in raw milk.

formed, which usually took place within 3 or 4 hr. None of the cultures examined in this way was agglutinated but all of them revealed that a single organism proliferated with the formation of a clump. Some of the strains forming long chains examined by this method gave similar results, the organisms forming long chains instead of clumps.

An attempt to identify the factors responsible for varying morphology in raw and heated milk

Heated milk

The initial work indicated that heating milk had an effect on the morphology of *Str. lactis*, therefore experiments were carried out to find the range of temperature influencing this variation.

Cultures showing long chain formation in raw 'sterile' milk were inoculated as previously described into milk heated to 44, 50, 64, 68 and 70° C. for 1 hr. and the lengths of chain compared. It was discovered that the milk heated to 44, 50 and 64° C. had little or no effect on the length of chain formed, but heating to 68 and 70° C. had a significant effect. As the maximum variation in morphology took place in milk heated to 70° C. for 1 hr. it was decided to adopt this time and temperature of heating the milk during all the experiments recorded in this paper except where otherwise stated.

This effect of heating milk to 70° C. for 1 hr. appears to be critical for the production of short chains, and as 70° C. is approaching the temperature for the denaturation of protein (5, 8) it was considered that protein denaturation may be responsible for short chain formation. This possibility is under further investigation and will be reported in due course.

Raw milk

During examination of the smears prepared for microscopical examination at hourly intervals from inoculated raw milk it was noted that all cultures producing long or short chains tended to have shorter chains at 5–6 hr. than at 3–4 hr. This shorter chain formation was at first thought to be due to disintegration of the long chains during the preparation of the smears, but as the organisms forming only short chains showed a similar tendency it was considered that some other factor, such as the by-products of growth of the organisms, was beginning to have some influence on the length of chain. The inoculated raw milks incubated for a longer time at 37° C. proved this supposition to be correct, as with the longer incubation time the chains gradually became shorter until at 24 hr. they were in the form of short chains or diplococci. The milk at the end of the 24 hr. incubation was always clotted and it was thought possible that the production of lactic acid by the organisms might be linked with this morphological alteration.

Pure lactic acid was added to raw, inoculated milk in varying amounts to find the effect on chain formation, but it was found that lactic acid added in amounts which did not inhibit the growth of organisms had no effect at all. On the other hand, experiments carried out on the effect on chain formation of acid produced by the organisms themselves are still in progress and are yielding conflicting results. In some cases long chains are formed when the natural acidity produced by the organisms is periodically neutralized, whereas in other cases the neutralization of the acid has no effect and short chains are formed which seems to indicate that some other by-product of bacterial metabolism may influence chain formation.

DISCUSSION

As the result of finding that different strains of *Str. lactis* varied in morphology in raw and heated milk, it was thought this variation might considerably affect the accuracy of the colony-count test when used for estimating the numbers of viable organisms present in milk, and might also account for heated milk and raw milk having the same methylene-blue reduction time, although the colony counts were very dissimilar.

Hobbs (5) reported the presence of an agglutinating factor in raw milk that could give similar results, but after a considerable amount of work carried out on the direct microscopical examination of the organisms at frequent intervals after inoculation, and during the logarithmic phase, we decided it was not an agglutinating factor responsible for the low colony counts on raw milk compared with heated milk, but the organisms were growing in the form of long chains or clumps in the early hours of incubation and were not broken up during the preparation of dilutions.

The T.M.C. on all raw milks inoculated with cultures of *Str. lactis* that produced long chains always gave lower results than the T.M.C. on the same cultures inoculated into heated milk. This consistently lower T.M.C. in the raw milk rather indicates the presence of a second factor responsible for the lower colony counts in raw milk than in heated milk. This factor could be the growth inhibitory factor present in raw milk reported by Hobbs (5), but our findings show that the main cause of the low colony counts was due to variations in morphology rather than the presence of a growth inhibitory factor.

SUMMARY

1. *Streptococcus lactis* cultures were found to vary considerably in their morphological characters when grown in raw and heated milk.

2. This variation in morphology was found to be the main cause for the difference in colony counts between inoculated raw milk and milk heated to 70° C. for 1 hr. over a 6 hr. incubation period at 37° C.

3. Similar T.M.C.'s on inoculated raw and heated milk during incubation at 37° C. explained why the methylene-blue reduction times were the same.

4. It is thought that denaturation of protein may be responsible for the production of short chains in heated milk.

5. The by-products of growth of *Str. lactis* are responsible for the production of short chains in raw milk after a long incubation time at 37° C.

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387. BACTERIOPHAGE IN TYPING LACTIC STREPTOCOCCI

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I. REVIEW OF THE PRESENT PROBLEMS

The occurrence of a bacteriophage, lysing starter organisms, was first reported by Whitehead & Cox (1). Since then many workers have shown that failure to attain the desired acidity in cheese and butter manufacture is often due to phage lysis of the starter (Nelson & Hammer (2); Babel (3); Johns & Katznelson (4, 5); Sutton (6); Yakovlev (7); Palladina, Peretz & Mazukevitch (8); Mazé (9); Josse (10); Pette (11); Anderson & Meanwell (12); Mattick, Nichols & Wolf (13)).

Whitehead & Hunter reviewed, up to 1945, their work on phage attack of cheese starters (14). Important advances in phage control were made only after they had found that phage was air-borne in the factory (15).

Nichols & Wolf (16) attempted, by means of phage affinities, to establish identity or relationship of strains from commercial starters. The component strains of a commercial starter were not always lysed by the same phages. Methods for control of phage attack in the factory, using the accumulated knowledge, have been recommended by Nichols (17).

The work of Meanwell (12, 18) first led to the idea of rotational use of starters. He used several mixed culture starters daily and immediately discontinued the use of any starter for which a dangerous concentration of phage was detected in the factory whey. Later he found that he could safely reintroduce into the factory, after an interval of several days, a starter which had previously been phaging. Mattick *et. al.* (13) used a similar rotation of mixed starters and were able to avoid dangerous accumulations of phage

('build up') for any particular starter in a factory. These workers have appreciated that phage action on a mixed strain starter may not be specific and that the interpretation of phage detection results may be complicated by the use of mixed starters, but smooth running in the cheese room without 'slowness' has been achieved.

The rotational use of a collection of unrelated strains rather than of mixed culture starters might be a solution to the phage problem. It would still guard against extensive phage attack and would simplify the detection of phage in the factory whey. Whitehead & Hunter, with their established preference for the daily use of the same single strain starter, have indeed reported (19) experiments in the use of a 4-day rotation of four different strains. More recently they have further modified their views and advocated a 4-day rotation of eight strains, using two of the strains daily (20).

After the first selection of a few basic strains, unrelated strains are difficult to find. The addition of a new strain, completely unrelated in phage-reaction to five or six original strains, involves numerous cross-phaging tests. The present work was undertaken in an attempt to facilitate the identification and establishment of the phage relationships of the strains in use and of newly isolated strains, which might be used for starter.

II. STRAINS OF LACTIC STREPTOCOCCI

1. SOURCES OF STRAINS

Approximately 450 strains of lactic streptococci have been selected from thousands collected during the last 6 years. They comprised two main groups: (i) 'cultivated' strains from commercial starters; (ii) 'wild' strains from sour milk.

(i) *'Cultivated' strains*

The first 'cultivated' strains isolated were from commercial starters which had been attacked by phage when used in the factory and the strains were lysed by phage in the whey. After plating the starter on yeast-dextrose agar (Y.D.A.*), colonies were picked into yeast-dextrose broth (Y.D.B.*) and any strains lysed by the corresponding filtered factory whey were purified by three successive platings on Y.D.A. (16). Initially most of these strains rapidly produced acid in milk.

It was later found to be preferable when isolating strains from starter to pick colonies into litmus milk and continue to work in milk culture. Some indication of the rate of acid production in milk, facilitating selection, was thus provided as soon as the strains were isolated.

Lactic streptococci have also been isolated from commercial starters for which no lytic phage was available at the time. They were selected for 'activity' as described for the 'wild' strains (Nichols & Hoyle (21)).

The starters, yielding 'cultivated' strains, were drawn from a wide field. In this country, Dairy Schools, cheese-making factories and commercial firms supplying powdered or liquid starter which originated from Denmark, Holland or U.S.A. contributed strains. Probably most of these starters once contained several strains, and many were undoubtedly of the same origin. Single-strain or mixed-strain starters have also been obtained directly or indirectly from the collections of other workers, including L. J. Meanwell (United Dairies, London), H. R. Whitehead and G. J. E. Hunter (New Zealand), F. J. Babel (U.S.A.), R. P. Myers (U.S.A.) and R. Wahl (France).

A few substrains, isolated from platings of strains, have also been retained for study.

* Y.D.B. 2% peptone, 1% lemco, 0·5% dextrose, 0·5% salt and 0·3% Yeastrel. Y.D.A. ditto + 2% agar.

(ii) *'Wild' strains*

These strains were obtained from milk during a search for lactic streptococci which could be used for starter. Details of methods of isolation and the basis on which the strains were selected in the first instance have appeared elsewhere (21).

After Hoyle & Nichols (22) had noticed that numbers of these 'wild' strains, selected for starters, inhibited the growth of similar streptococci, the final selection was made after they had been tested for inhibitory power. A strain was finally discarded, if the addition of 10% of heated whey retarded acid development by the test organism.

As reported previously (21), silage was not found to be a suitable source of starter strains and Pette (23) recorded that *Streptococcus lactis* was never found in grass silage, except when added initially in whey.

2. LOSS OF ACTIVITY

One of the essential properties of a starter strain is ability to ferment lactose rapidly under the conditions of cheese manufacture, but the production of acidity in milk equal to 0·6–1·0% lactic acid in 6 hr. at temperatures from 30 to 40° C. is exceptional and the number of such strains is limited. It is important, therefore, to retain their activity.

It is customary to subculture starter cultures every 24 hr. and to incubate at a low temperature. Sherman & Hodge (24) found that by transferring cultures at 12 instead of 24 hr. intervals the total acid production was reduced. They thought that, for the two cultures tested, the fastest growing organisms were producing the least total acid and these were secured in largest relative proportions by frequent transfers.

Dahlberg & Ferris (25) questioned the value of regular subculture but found that infrequent subculture resulted in slower starter and poor cheese.

By plating and picking colonies, Harriman & Hammer (26) showed that certain *Str. lactis* cultures, which rapidly coagulated milk, could be divided into 'rapid' and 'slow' strains, but that strains from a 'slow' culture were all slow acid formers.

Mazé & Mazé (27) observed that some lactic streptococci from milk quickly lost their original power of acid production when subcultured in milk but others retained it for years. The proportion of 'slow' types in the active and stable cultures varied from 60 to 90%, and the active types quickly dissociated into 'fast' and 'slow' strains on subculture.

Rapid acid formation may be lost during repeated subculture in milk, and Hunter (28) combated this by frequent plating and selection of active colonies.

Over a number of years some general information on acid production has been secured. It supports some of the observations of other workers, but since the problem involves the methods of storage and rejuvenation of cultures, this matter will be discussed first.

3. STORAGE OF STARTER AND STRAINS AND METHODS OF REJUVENATION

Two methods of storage have been used for maintaining the viability and rate of acid formation for long periods.

(i) *Storage in litmus chalk milk at* 1–5° *C.*

Barthel (29) found that organisms of the *Str. lactis* group could be kept alive for long periods in milk to which chalk had been added. Milk is slowly acidified at low temperatures and an added indicator, such as litmus, facilitates observations during storage.

In the earlier work, actively growing 24 hr. milk cultures of the organism to be stored were inoculated ($\frac{1}{20}$ ml.) into tubes of litmus chalk milk (10 ml.)* and incubated at 30° C. until the medium was on the point of coagulation. Thereafter they were kept at not more than 5° C. After 3–6 months the tubes were incubated at 30° C. and subcultured daily in litmus milk. After four to seven daily subcultures, when growth was active, cultures were made in chalk litmus milk and returned to the refrigerator.

The following modifications of this method, which reduced failures to grow actively and increased the period of storage, were finally adopted. Litmus chalk milk was heavily inoculated ($\frac{3}{4}$ ml.) with a 24 hr. culture, well mixed, incubated at 30° C. or left in the laboratory until reduction of the litmus had just begun and then refrigerated. Before rejuvenation, the medium was thoroughly mixed, using a pipette, and the tubes placed at laboratory temperature or 22° C. until signs of growth, no matter how slight, were indicated by changes in the litmus. The cultures were then subcultured into litmus milk and incubated at 22° C. When there was no apparent alteration, even for several days after a culture had been removed from the ice chest, revival was assisted by adding sterilized N/10 soda or acid solution to bring the reaction nearer to neutrality. (If no growth occurs during storage, litmus chalk milk may become alkaline, hence the necessity for the addition of acid.) It has been possible to store lactic streptococci, using these modifications, in chalk litmus milk for a least a year without loss of activity.

(ii) *Storage at laboratory temperature after drying cultures* in vacuo

It is generally accepted that 'freeze-drying' is the best method of preserving the properties of bacteria for long periods. Mattick & Rogers (30) found that *Str. cremoris* was still fully vigorous after it had been dried and stored at room temperature for 11 months.

An adaptation of Swift's method (31), suggested by Rhodes (32), was used for drying cultures of lactic streptococci. Growth from a 24 hr. culture on Y.D.A. was mixed with horse serum to give a milky suspension. A loopful of the suspension was placed on the side of a small plugged tube which was introduced inside a larger outer tube containing a small quantity of P_2O_5. The outer tube was drawn out in the blow-flame to give a short ($1\frac{1}{2}$ in.) length of narrow-bored tubing, which was quickly evacuated (1 min.) and sealed at the constriction. The evacuated tubes were stored at room temperature.

When rejuvenating the cultures, after confirming that the vacuum was still satisfactory, the end of the outer tube was broken and 1–2 ml. of litmus milk was added to the inner tube. The stain of the dried culture on the side of the tube was then rubbed with an inoculating loop and the tube incubated at 22° C. Usually the strains showed active growth within 24–48 hr. and thereafter were subcultured daily in milk.

Although only about thirty tubes could be dried in an hour this method was preferable to those in which the tube actually containing the culture was sealed off, since the outer tube could be broken without danger of contaminating the culture.

Most of the strains referred to earlier have been dried and stored *in vacuo*. The strains have been stored dry for 3–$3\frac{1}{2}$ years, but no failures in rejuvenation have yet been recorded.

Three commercial starters and seven strains, three *Str. lactis* and four *Str. cremoris*, have been dried. Special note has been made of rejuvenation and of changes in rate of acid production after lengthening intervals of storage. They were dried as described above, with certain modifications.

* Separated milk + 1·0% litmus solution and 0·5% calcium carbonate.

The drying tubes received one of the following:

I. 24 hr. culture grown on Y.D.A. + horse serum.
II. 24 hr. litmus milk culture + equal quantity of horse serum.
III. 24 hr. litmus milk culture alone.
IV. 24 hr. neutralized litmus milk culture + equal quantity of horse serum.
V. 24 hr. neutralized litmus milk culture alone.

The dried cultures were revived in the usual way at 22° C. and the activity determined after 3 and 6 (or 7) days. Typical results for *Str. cremoris* 950/5 are given in Table 1.

Of 405 dried cultures examined over a period of 2 years only six have not been recovered. The medium in which they were dried had little effect on survival and there was no appreciable change in activity on storage but there was a suggestion that drying from a 24 hr. culture of the strain in milk was less satisfactory. All the cultures produced acidity at a normal rate 6–7 days after rejuvenation, most of them within 3 days.

Table 1. *'Activity' of strain* 950/5 *revived after drying and storing* in vacuo

	Period of storage															
	13 days		3 weeks		5 weeks		8 weeks		4 months		8 months		16 months		26 months	
	Days after commencing to revive strain															
Dried in	3	6–7	3	6–7	3	6–7	3	6–7	3	6–7	3	6–7	3	6–7	3	6–7
I. Horse serum	5·5	5·5	—	5·1	5·0	5·0	4·8	5·3	6·1	5·2	5·1	5·1	6·8	5·5	5·1	5·4
II. Milk + horse serum	5·2	5·3	5·1	5·5	5·4	5·2	4·3	5·4	5·6	5·2	5·2	5·1	6·3	5·3	5·6	5·1
III. Milk	—	—	5·0	5·2	5·1	5·0	4·6	5·1	5·7	5·2	5·3	5·5	5·8	5·7	5·7	5·4
IV. Neutralized milk + horse serum	5·3	5·4	5·3	5·2	5·1	5·1	4·5	5·2	5·0	5·2	5·2	5·3	6·0	5·3	6·0	5·2
V. Neutralized milk	5·0	5·1	4·9	5·0	5·4	5·3	4·5	5·2	6·5	5·4	5·1	5·4	4·9	5·4	4·9	5·3

The results given are titration figures of the 'activity' tests, i.e. no. of ml. N/10-NaOH required to neutralize the acidity of 10 ml. of milk inoculated with 1% of cultures after 6 hr. incubation at 30° C.
'Activity' of 950/5 when dried (May 1946) 5·4.

The following points of interest in respect of the preservation of activity in starter organisms, have emerged in the course of some years.

(1) For milk cultures an incubation temperature of 22° C. is preferable to 30° C. The acid-producing power is less impaired at the lower temperature. When several strains were subcultured daily for some months in milk medium at these temperatures, the deterioration, long suspected, in rate of acid production at 30° C., was confirmed.

(2) Some 'fast' strains whether from starter or elsewhere have lost the property within a few days, although the original stock culture, subcultured under the same optimum conditions for preserving acid production, has remained unchanged.

(3) The property of producing acid rapidly in milk has, with one possible exception, never been acquired by 'slow' strains during daily transfer in milk. Usually such strains, because of the objects of the work, were quickly discarded and opportunities to observe increases in rate of acid production in 'slow' strains were limited, but would have been noticed, if they had occurred, amongst the numerous 'slow' strains subcultured for short periods. When a 'fast' strain loses its ability to acidify milk rapidly the resulting 'slow' strain does not appear to differ from the original 'fast' strain in any other respect and, therefore, it does not seem unreasonable to expect changes to occur in the opposite direction (i.e. 'slow' to 'fast'). Reduction in rate of acid production in milk often occurs when a 'fast' strain is attacked by phage—the resulting resistant secondary strain being 'slower' than the original strain.

(4) Large numbers of colonies had to be picked from some of the 'fast' starters or strains before one 'fast' culture could be secured. In isolating the 'fast' strains 725/33, 950/5 and 951/6 from the starters 725, 950 and 951 respectively, at least fifty colonies from both streaked and poured plates were picked and found to be 'slow'. No 'fast' strain was obtained from about the same number of colonies picked from starters 952 and 975. It thus became clear that 'fast' strains did not always predominate in 'fast' starters (24 hr.) The tendency had been to regard 'fast' acid production in milk as associated with large numbers or rapid proliferation of 'fast', compared with 'slow', strains. The development of 'fast' and 'slow' strains in a 'fast' culture on daily transfer in milk at 22° C. was investigated.

Two 'fast' strains of *Str. cremoris*, 1 P 5 and 144 F, were chosen. Each organism was plated on Y.D.A. and twelve colonies picked into Y.D.B. as a preliminary test had shown that at least one 'fast' culture (substrain) could be expected among twelve colonies from platings of 1 P 5 or 144 F. The 24 hr. Y.D.B. cultures were inoculated into separated milk to give (using Brown's opacity comparator) approximately equal numbers of organisms in the milk, which was incubated at 30° C. At intervals the milk was plated to determine the number of organisms present and tested for acidity. From culture 1 P 5 two 'fast' substrains 1 P 5/1 and 1 P 5/6 and from 144 F one 'fast' substrain 144 F/4 were picked. The results, summarized in Table 2, indicate that in the 'fast' strains the rate of proliferation was greater than with the 'slow' acid-producing strains, although they had been picked only 24 hr. previously from the same culture. From these results it is not possible to predict whether the strains which produced acidity most quickly in milk will die out more rapidly than 'slow' strains.

With strain 144 F/4, the maximum number of organisms was attained between 12 and 24 hr. after inoculation, whereas with 1 P 5/1 and 1 P 5/6 the numbers present were highest 24 hr. after inoculation.

Table 2. *Relationship of acid production rate and numbers of organisms in 'fast' and 'slow' strains*

A		'Fast' substrains (average of 2)		'Slow' substrains (average of 10)	
Source of substrains	Time after inoculation (hr.)	Acidity*	Plate count per ml. (millions)	Acidity*	Plate count per ml. (millions)
1P5	0	2·1	2·2	2·1	1·7
	5	3·0	250	2·7	70
	6½	3·6	600	3·0	110
	7¼	5·4	600	3·4	220
	12	7·5	1600	4·5	310
	24	8·7	4000	6·6	470

B		'Fast' substrain (one only)		'Slow' substrains (average of 11)	
Source of substrains	Time after inoculation (hr.)	Acidity*	Plate count per ml. (millions)	Acidity*	Plate count per ml. (millions)
144F	0	2·1	0·5	2·1	0·4
	5½	3·0	80	2·7	36
	7½	5·0	250	3·4	50
	12	7·9	1400	4·1	150
	24	8·4	140	5·6	170

* ml. N/10-NaOH required to neutralize 10 ml. of inoculated milk.

Table 3

Substrain 1 P 5/1 isolated 4. xi. 46			Substrain 144 F/4 isolated 26. x. 46		
Daily sub-culture before picking (weeks)	No. colonies picked		Daily sub-culture before picking (weeks)	No. colonies picked	
	Total	'Fast'		Total	'Fast'
0	24	24	0	24	24
1	9	7	1	12	11
9½	10	4	2	12	11
13	9	4	6	12	9
17	10	2	9½	10	9
23	12	0	14	9	6
31	10	4	18	10	6
38	12	3	24	12	8
51	12	4	32	10	6
54	12	1	39	12	8
77	12	3	51	12	11
89	20	7	79	12	10
93	20	8	91	20	10
			94	20	11

Two 'fast' substrains 1 P 5/1 and 144 F/4 were, from the time of isolation, subcultured daily in milk and incubated at 22° C. for a period of 2 years and have retained their original 'activity'. At intervals 24 hr. cultures have been plated and colonies picked. The cultures isolated were classified as 'fast' or 'slow' (Table 3). During the first 18 months of daily subculture the substrain 144 F/4 was still composed mainly of 'fast' strains, whereas 'slow' strains quickly predominated in 1 P 5/1. After further subculture daily half the colonies picked from 144 F/4 were 'fast' and the proportion of 'fast' colonies obtained from 1 P 5/1 increased.

Many strains, especially those now some 6 years old, are no longer 'fast' in milk, but the best conditions of transfer and storage for maintaining fast acid production were not at first fully appreciated. Plating and picking active strains to maintain vitality (Hunter (28)) have not been extensively used. It would be an advantage to plate and repick active strains from an unstable strain like 1 P 5/1 because with the rapid development of the 'slow' substrains there is danger that the 'fast' strains may be lost. The results with 144 F/4 show that such a procedure is not necessary with all strains even though subcultured daily in milk for long periods, and Mazé & Mazé (27) concur in this opinion.

III. THE IDENTITY OF STREPTOCOCCI FROM STARTER AND OF STREPTOCOCCI, SUITABLE FOR USE AS STARTER, ISOLATED FROM MILK

Earlier work on the identification of strains of lactic streptococci mentioned in the last section has been published in detail by Nichols & Hoyle (21). The following is a brief summary of this work with further data on the isolation of *Str. cremoris* from raw milk. It was possible that clues to the susceptibility of the different strains to phage lysis might emerge after the identity of the strains of lactic streptococci had been established.

The strains were identified by tests based on work of Sherman (33), Hunter (34) and Abd-El-Malek & Gibson (35). All the 'cultivated' strains isolated from commercial starters were identified as *Str. cremoris*. With two exceptions, the 'wild' strains from sour milk proved to be *Str. lactis*. The detailed tests applied and their value in the identification of *Str. cremoris* and *Str. lactis* strains have been discussed (21). Three differential tests, namely: (i) action in litmus milk, (ii) hydrolysis of arginine, and (iii) ability to grow in litmus milk

at 40° C., have been claimed to be sufficient to distinguish *Str. lactis* from *Str. cremoris*. The strains more recently added to the collection have been identified by these three tests.

Str. cremoris strains were found in 43% of the raw milk samples examined by Abd-El-Malek & Gibson (35). An additional forty-four samples, eleven from large bulks of milk (usually tankers) and thirty-three farm samples, were examined in this Institute especially for the presence of *Str. cremoris*, since none were found amongst the original collection of 'wild' strains of lactic streptococci from milk. Only five strains of *Str. cremoris*, four with a good activity and one which produced acid in milk slowly, from over 200 colonies picked, were obtained and all were from large bulks of milk. As slow acid-producing strains were usually discarded at an early stage other *Str. cremoris* strains may have been picked, but not retained for identification. The rarity of *Str. cremoris* strains may possibly be due to the treatment of the milk samples before plating.

IV. BACTERIOPHAGES FOR THE LACTIC STREPTOCOCCI

1. SOURCES OF PHAGE—DAIRY PRODUCTS

Since a phage proliferates only in the presence of the organisms susceptible to lysis by that phage it is to be expected that phages will be found where the host bacteria occur. Possible sources of phages for the lactic streptococci might, therefore, be the same as those of the organisms themselves, viz. milk, dairy products and by-products, dairy utensils and the environment of dairies.

The contents of the vat during cheese-making may be considered as a massive culture of lactic streptococci, and most workers on 'slow' cheese problems have sought phages for starter organisms in the cheese whey. Whitehead & Hunter (36) noted the presence of phage in whey from cheese vats, even where acidity developed normally in the curd. Other workers (3, 4, 6, 12, 13) have obtained bacteriophages, capable of lysing strains of lactic streptococci, from cheese-vat wheys.

In seeking phages for strains used in the present work factory whey was the first source extensively explored. During the 1942–4 seasons Nichols & Wolf isolated phages for many *Str. cremoris* strains from factory wheys (16). As additions were made to the collection of lactic streptococci suitable for starter many strains which were not lysed by any of the phages already isolated were obtained. The methods used and sources explored in an attempt to isolate phages for these strains are given below.

(i) *Factory whey*

(*a*) *As a source of phage for starters in current use*

The methods used for the isolation and purification of the phages were similar to those given by Nichols & Wolf in obtaining phages from whey (16). Each starter was tested by an activity test for susceptibility to phage in the corresponding whey. Any starter which was, on adding whey, retarded in acid production by 10% or more, was regarded as 'phaging' and was plated on Y.D.A. Twelve to twenty-four colonies were picked into Y.D.B. and tested against the filtered whey. The strains susceptible to phage lysis were purified by two further platings on Y.D.A. After each stage of strain-purification, isolated colonies were picked into Y.D.B. and, after growth, were tested against the filtered whey to confirm that strains subject to lysis by the phage in the whey had been picked in each instance.

The corresponding phage was isolated and purified by three successive pickings of plaques from Y.D.A. plates, using the purified strain as host. Plaques were picked into Y.D.B. and, after clearing, the broth was passed through a Seitz filter.

When attempting to isolate certain phages, although their presence was indicated by the activity test and by plaques on Y.D.A., no clearing was observed when the plaques were picked into Y.D.B. If such plaques were picked into litmus milk phage could be shown to be active. The phaging cultures were then acidified with sterile dilute acid (HCl) until the casein was precipitated, centrifuged at approx. 2000 r.p.m. for 5 min. and the supernatant passed through a Seitz filter.

In all, phages for 147 strains were added to the collection from factory wheys between 1942 and 1948. Many of the commercial starters were of unknown constitution and it is probable that many similar strains were isolated. Many of the phages may therefore be identical.

(b) As a source of phage for any strains of lactic streptococci

As the collection of lactic streptococci was augmented many strains of *Str. lactis* and *Str. cremoris* were added before they had been used for factory cheese-making. Thus, there was no opportunity to obtain phages, active against them, from the corresponding factory wheys. Phages for these strains were sought in random samples of whey collected from different factories. It was unlikely that the whey would contain a sufficiently high concentration of phage, specific for any one of these strains, to affect the titration figure in the activity test by 10%. The concentration of any phage, capable of lysing the strain under test, was therefore first increased ('built up') by growing with the appropriate organism.

Similar methods of 'feeding' have been used by many workers isolating bacteriophages for other organisms, e.g. Evans & Sockrider (37), Asheshov (38). Sutton (6) applied an enrichment method to obtain phage, for a strain of *Str. cremoris*, from whey, and Hunter (34), in isolating phages for lactic streptococci from factory whey supplies, 'built up' the strength of the phage by 'feeding' on the appropriate organism.

Method for 'building up' phage concentration by 'feeding'. Two tubes containing 10 ml. litmus milk were seeded with the selected organism (usual inoculum two drops $\frac{1}{10}$ dilution of a 24 hr. culture in $\frac{1}{4}$ strength Ringer's solution). One, the control tube, received no further inoculum; to the other, the + tube, approx. $\frac{1}{4}$ ml. Seitz-filtered whey was added. Both tubes were incubated for 24 hr. at 22° C. after which the milk in both tubes had usually coagulated. Two further tubes of litmus milk were then seeded, the control tube receiving two drops from the previous day's control tube, the other, one drop from the control and one from the + tube. This process was repeated daily for five or six transfers, if necessary, but usually if any phage action occurred it was apparent after two or three transfers. The + tube then showed little change or only reduction of the litmus compared with the control tube in which the litmus was reduced and the milk coagulated. When such differences between the two tubes were noted the contents of the + tube were tested for the presence of phage either by the activity test or by plaque formation on agar using dilutions of the material usually without previous filtration. The phage was then purified as indicated above.

Twelve whey samples from four factories, two using heat-treated milk and two raw milk for cheese-making, together with twenty-two old filtered factory wheys, which had been stored in the refrigerator for 5 or 6 years, were examined as sources of phage. The indicator

strains used were mainly *Str. lactis*, many newly isolated from milk; a few were recent isolations of *Str. cremoris* strains. After 'building up' the phage concentration many positive reactions were detected by the activity test; from these, phages were propagated and purified on twenty-five strains, twenty-one *Str. lactis* and four *Str. cremoris* strains. The presence of phage was indicated, by the activity test, for a further six *Str. lactis* strains but no phage was purified. Plaques were obtained on Y.D.A., but purification was difficult owing to the minute size of the plaques. Moreover, it was not possible to obtain a filtrate containing a high concentration of phage, even where plaque counts in the unfiltered cultures were high, since most of the phage particles appeared to have been removed by Seitz filtration.

Many of the strains of *Str. lactis*, for which phage was 'built up' from a collection of wheys, were recent isolations from sour milk. Nelson, Harriman & Hammer (39) were unable to obtain lytic filtrates active against such strains, but Anderson & Meanwell (12) found that freshly isolated strains of *Str. lactis* introduced into the factory as starter were readily attacked by phage. In the present work no great difficulty in securing phages capable of lysing these strains was encountered.

(ii) *Bulk milk*

Phages for some strains of lactic streptococci which appeared to be unrelated to the strains in current use in the factory were obtained for factory whey; these phages may have been present in the bulk milk before it reached the cheese factory.

Yakovlev (7) reported that milk was not a source of phages for *Str. cremoris* strains, but Palladina *et al.* (8) found the principle source of bacteriophage for the lactic streptococci to be mixed city milk. Mazé suggested that raw milk coming into the cheese factory might be a source of phage (40), but stated later that fresh milk at the farm does not normally contain phages (41). Johns (42) observed phage in the milk supply, but the constituent individual cow samples yielded no phage. He reported phage in the milk sampled from the vat at the dairy, but failed to realize that phage from previous outbreaks of 'slow' cheese would still be present on utensils and in the air at the dairy.

Whitehead & Hunter (19) consider milk delivered to the factory in churns, also used to transport whey, to be a source for the dangerously high concentrations of phage which sometimes occur.

As indicated earlier, phages in milk capable of lysing strains of lactic streptococci would be expected to be associated with *Str. lactis* or *Str. cremoris* strains, on which they proliferated, in that milk. Single cow samples would rarely contain lactic streptococci, since they are not normally present in the udder, and phages for *Str. lactis* or *Str. cremoris* strains in single cow samples are unlikely to occur. In a sample of milk, taken from a large bulk, lactic streptococci would form a significant part of the flora and phages active against them might be detectable; such bulk milk was investigated as a source of phages active against the lactic streptococci.

Twelve bulk milk samples, independent of cheese factories, were examined. Three samples consisted of a mixture of six different farm samples; the others were from large static tanks or tankers. All the samples were acidified, centrifuged, Seitz filtered and the resulting whey 'fed' to eighty strains from the collection. Equal numbers of *Str. lactis* and *Str. cremoris* strains were used and 'whey' from the bulk milk samples 'fed' to the strains in litmus milk, not usually beyond the third subculture. About 450 different whey-

organism combinations were tested. When the presence of phage was indicated it was confirmed by plaque formation on Y.D.A. Phages were obtained for thirty-six strains, eleven *Str. lactis* and twenty-five *Str. cremoris*.

(iii) *Cheese-factory products*

(*a*) *Cheese*

Although failure to recover phage from a 'slow' cheese after only 18 days' ripening was reported by Johns & Katznelson (4), other workers do not seem to have experienced this difficulty. Nichols & Wolf (43) recovered phages from cheese after storage for periods of up to 12 months and noticed little decrease in phage concentration during storage. Seven of the phages in our collection were isolated by them from cheese. Hunter (34), in isolating phages for the lactic streptococci, found cheese to be a very convenient source.

(*b*) *Whey powder*

Spray-dried whey powder, reconstituted by adding one part whey powder to five parts ¼ strength Ringer's solution, was examined as a further source of phages for starter organisms. The mixture was shaken well with glass beads, centrifuged lightly and Seitz filtered. Addition of the filtrate slightly retarded acid production in certain starters. The phage concentration was 'built up' by 'feeding' the extract on susceptible strains and purified in the usual manner. Phages active against two *Str. cremoris* strains were obtained from this source.

2. INVESTIGATIONS OF OTHER POSSIBLE SOURCES OF PHAGE

(i) *Enterococci*

The enterococci have their origin mainly in the intestine of warm-blooded animals (33) and may, therefore, contaminate milk. The hardiness of these streptococci, together with their wide range of growth temperatures, makes possible their survival and multiplication under adverse conditions in nature. Phages for the enterococci, perhaps present in cow-dung, might enter the milk intended for cheese-making at the farm. If these phages could attack the strains of lactic streptococci, they could constitute a constant source of danger to starter.

Three phages for enterococci, *h*,* *d* and *wm* were added to Y.D.B. and milk cultures of strains of lactic streptococci. Altogether 182 tests with different combinations of the three phages and strains of *Str. lactis* and *Str. cremoris* in broth were made and 132 activity tests were carried out in milk. There was no indication that these phages for enterococci, even when used in concentrated suspension, could attack any strains of lactic streptococci. Attempts were also made to promote phage attack by adding a mixture of four phages for *Str. cremoris* and one for *Str. lactis* to twenty *Str. faecalis* strains.

The collection of enterococci consisted of three old stock cultures, two isolated from cheese, two from milk and the remainder recently obtained from cow-dung. A further attempt, without success, was made with a mixture of five *Str. cremoris* phages and eight MacNeal strains of enterococci to promote phage attack of the enterococci.

* Phage *h* obtained from Sir Thomas Houston, Belfast. Isolated from a case of ulcerative colitis. Phage *d* obtained from Dr Verder from the Alice Evans collection (44), stain 693 isolated from faeces of case of intestinal haemorrhages, phage *wm* obtained from Dr Ward J. MacNeal, Columbia University, U.S.A. Also eight strains of enterococci lysed by phage *wm*.

Attempts were also made to isolate phages, from cow-dung or from the drainage of a manure heap, for the collection of strains of enterococci detailed in the previous paragraph. Samples of dung or manure heap effluent were usually mixed with equal quantities of broth and Seitz filtered. The test strains were 'seeded' into milk and broth media and the filtered preparations of cow-dung or manure heap effluent added as a small (loopful) or large (25 ml.) inoculum. For some tests the dung preparation or effluent was unfiltered. The strains were serially subcultured four or more times in the same manner as for 'building up' a phage from whey (p. 175) with or without Seitz filtering the + tube after each 'feeding' and tested by addition of filtrate from the final + tube in milk or broth for phage action. No phage was obtained for any of the strains of enterococci tested even though many had been isolated from cow-dung.

Whilst this work was in progress Hunter (34) noted that all *Str. faecalis* cultures which he had tested were completely insensitive to phages which attacked his group I *Str. cremoris*, or his group II *Str. lactis*. Graham & Bartley (45) have reported that no phage action occurred when three phages for the enterococci were spotted on to two strains of *Str. lactis* inoculated on the surface of agar. They concluded that *Str. lactis* was unrelated to the enterococci.

The work reported in the present paper is rather slight, but in view of the findings of these other workers it seemed unlikely that a connexion between phages and strains of the enterococci and of the lactic streptococci would be established. Therefore no attempts to 'build up' phages for the lactic streptococci on strains of enterococci or vice versa were made. Solid media for phage detection as described by Fisk (46) were also not used. There was, moreover, no opportunity to test the action of phages for strains of saprophytic enterococci against the lactic streptococci.

(ii) *Farmyard manure*

Many phages, including streptococcal phages, have been isolated from sewage (37). Cow-dung and pig-faeces may for obvious reasons be sources of phages for the lactic streptococci and, if this is so, milk may be contaminated with phages at the farm.

(*a*) *Cow-dung.* In attempting to isolate phages, it was desirable, owing to the complexity of the dung flora, to work with filtrates prepared from dung, assuming that phages developed in the dung or multiplying on the flora, could be detected after filtration. A control experiment showed that phage added to dung and pig-faeces could easily be recovered from the Seitz-filtrate.

An attempt was then made to obtain from cow-dung phages for twenty-three strains of *Str. cremoris* but with no success.

(*b*) *Pig-faeces.* Similarly, no phages for five dissimilar strains of *Str. cremoris* could be obtained from pig-faeces.

Mazé (47) suggested that at a cheese factory where starters were attacked by phage, an adjacent piggery was responsible. He recovered from pig excreta phages for starter strains used in the neighbouring factory. He concluded that they originated in the whey consumed by the pigs and survived in the intestine for long periods. Later he believed that phages originated in the intestines of pigs which had had whey or separated milk in their diet. He tried to reproduce in artificial media (48) the conditions in the large intestine. The results are not convincing, but a non-specific phage of low activity for the lactic streptococci appears to have been isolated. He found that a lactic strain consumed by humans could

not be recovered from the faeces, nor could a phage, capable of lysing the strain consumed be detected (49), but he showed (41) that phages could be recovered from the faeces for several days after consumption of cheese containing them.

Whilst some of the statements made by Mazé may be debatable, it seemed worth while to pursue the thesis that phages may arise in the intestine of pigs. At the National Institute for Research in Dairying the presence of an isolated piggery, some miles from cheese factories, made it possible to conduct the following pig-feeding experiments without danger.

(1) For 10 weeks before slaughter a pig received up to 300 ml. per day of an active culture of *Str. cremoris* and daily for 1 week up to 10 ml. of a broth suspension of the homologous phage.

Repeated examinations of Seitz-filtrates from the faeces, mixed with Y.D.B. or water, and from the stomach and intestine contents after autopsy failed to reveal the phage.

(2) Two pigs received, over some 19 weeks, up to 300 ml. daily of another strain of *Str. cremoris*, 144 F. Three days before slaughter a further strain, 1 P 5, with its homologous phage, was also fed.

Examinations similar to those in (1) were consistently negative; no phage for 144 F or 1 P 5 was recovered: but when strains 144 F and 1 P 5 were grown with small quantities of post-mortem material, without filtration, phages for both were isolated. Mazé (47) also reported the recovery of phages from the faeces of pigs kept at a cheese factory, and the isolation of phage 1 *p* 5 was thus not unexpected. The presence of so dissimilar a phage as 144*f* is difficult to explain, particularly as the observation could not be repeated by feeding two further pigs and examining unfiltered faeces.

(3) No phage for strain 144 F was recovered from faeces or gut contents at autopsy after feeding this strain to two pigs for 4 weeks before slaughter.

(4) Four more pigs received milk cultures of 144 F for 11 weeks and 1 P 5 for 3 days before slaughter. After 1 week phages for 144 F were recovered from all the samples examined. The phage could not be found in the feeding stuff stored in the piggery.

(5) Two pigs were given 250 ml. daily of a milk culture of five strains of lactic streptococci, but no phages could be recovered. Thus for seven strains fed to pigs during the experiments, only one phage could be isolated. It therefore seems that though a phage may persist in a piggery or in the gut of a pig for some time the claim of Mazé (47) that the latter is a usual source of phage can hardly be sustained by these results.

(iii) *Lysogenic strains*

Since Lisbonne & Carrère (50) first described the ability of a *Bact. coli* strain to release a phage active on '*Shiga bacillus*' without contact with pre-existent phage, the conception of phage carrying, or phage and bacterium living symbiotically, without the occurrence of lysis, has been recognized in other groups of bacteria.

Burnet (51), working with *Salmonella* strains, reported that 75% of the strains showed 'some degree of lysogenicity toward one or other indicator organism'. He grew the strain to be tested with the indicator strain in broth and after 20–24 hr. heat treated (56° C. for 30 min.) the mixture to kill the bacteria. The heated culture was spread on agar, previously smeared with the indicator strain, in order to test for phage lysis. Burnet considers that the limitations of this method for obtaining lysogenic strains lie in the difficulty of finding suitable indicator strains.

If lysogenic strains occurred amongst the lactic streptococci they would greatly influence the selection of starter strains, especially where mixtures of two or three strains were to be used for cheese-making. A search was made amongst our collection of lactic streptococci for lysogenic strains and, as no indicator strains had yet been identified, the cultures were chosen at random. Pairs of strains were grown together in Y.D.B. and, after 24 hr. at 30° C., were Seitz filtered. A heavy inoculum of the filtrate (2–4 ml.) was added to two tubes containing 10 ml. Y.D.B., each seeded with one of the two strains originally grown together. The tubes were incubated at 30° C. and observed for phage lysis. Over 300 different pairs of *Str. cremoris* and *Str. lactis* were grown together, the combinations are summarized below:

Str. cremoris + *Str. cremoris*	212
Str. cremoris + *Str. lactis*	88
Str. lactis + *Str. lactis*	12

In all those instances, when either organism failed to grow in the presence of the filtrate, further investigation showed that failure was due to the presence of an inhibitory substance (22) produced by the other organism and not to phage action. No attempts were made to 'build up' a phage from any of the filtrates.

Thirteen strains of *Str. cremoris* were examined for lysogenicity, as suggested by Fisk (46) for staphylococci, by smearing each strain in turn on an agar plate and 'spotting' the remaining twelve strains on to the smeared plates. Among the limited number of cultures used, no lysogenic strains were detected. Before continuing with the search for lysogenic strains, it was necessary to know which strains in the collection could produce a substance inhibitory to the growth of similar strains (22). The search for lysogenic strains, on these lines, was discontinued at this point.

More recently, a culture of *Str. lactis* (C 30), whilst being used to demonstrate the lytic properties of certain phages, produced on the surface of agar a mat of growth containing innumerable plaques. The strain retained this characteristic from generation to generation but all attempts to 'build up' the phage from these plaques failed. A further search might yield a suitable indicator strain on which the phage could be propagated and thus unequivocably show strain C 30 to be lysogenic. It is, therefore, possible, as indeed might be expected, that lysogenic strains of lactic streptococci exist.

Phage-carrying strains of lactic streptococci have been reported by Hunter (52) but, since they have been shown not to be truly lysogenic, their occurrence and significance will be discussed later (p. 193).

(iv) *'Slow' mutants from 'fast' cultures*

It has been noted previously that on plating pure cultures of lactic streptococci, which rapidly coagulate milk, the resulting cultures could be divided into 'fast' and 'slow' strains (pp. 169 and 173). The ability of 'fast' strains to produce acid rapidly in milk is sometimes lost on daily subculture in milk (pp. 171–173). Mazé & Mazé (27) attribute to the 'slow' strains the property of delaying acidification of milk and compare them to 'slow' resistant strains formed after lysis of a 'fast' pure culture in milk by bacteriophage. They claim that this delay in acid production of 'fast' cultures is due to the presence of a weak, non-specific phage associated with the phage-resistant and degenerate ('slow') strains.

'Slow' and 'fast' strains were picked from plates of 'fast' *Str. cremoris* strains 1 P 5 and 144 F in order to detect such a phenomenon. Activity tests (16) were set up by using a 1% inoculum of a 'fast' strain (control) and adding two drops ($\frac{1}{10}$ ml.) of the 'slow'

strain to the second bottle; the acidities of the two milks were compared after incubation. All the 'slow' strains obtained from both cultures were thus tested with all the 'fast' strains obtained. None of the 'fast' strains was delayed in acid production on adding $\frac{1}{10}$ ml. of any 'slow' strain. Even after attempting to 'build up' the concentration of any phage present by 'feeding' the 'slow' strains to the 'fast' strains for several generations no delay in acid production could be detected. This source did not appear to be a profitable one from which to isolate phages for the lactic streptococci.

3. ADAPTED PHAGES

A large number of Seitz-filtered phages was collected from the sources previously mentioned. They were tested on the collection of strains so that additional phages, for strains not lysed by any of the available phages, could be sought. Lytic filtrates prepared at different times, for any one strain, varied in titre. It was, therefore, necessary to have some standard for comparison of the phage concentration in different filtrates of the same phage and for comparing the reactions of several phages against any one strain, or one phage against several strains. The concentration of phage in each lysate was estimated, using the strain on which the phage was originally propagated.

Estimation of phage concentration

The method is a modification of that used by Wilson & Atkinson (53) in titration of lytic filtrates for strains of staphylococci. The filtered phage suspension was diluted 1/10 to 1/100,000 in $\frac{1}{4}$ strength Ringer's solution. Two drops ($\frac{1}{10}$ ml.) of a 24 hr. culture of the homologous strain were spread evenly over an agar plate, previously dried, and after the surface of the agar had again dried it was 'spotted' with a standard loopful of each dilution of the phage suspension. Plates were incubated overnight at 30° C. and read the following morning. The highest dilution giving confluent lysis was regarded as the test dilution or critical dilution. This test dilution was then used in determining phage reactions for other strains on Y.D.A.

Preparation of lytic filtrates for previously resistant strains

Strains which showed no phage reaction with test dilutions of available phage suspensions were tested with undiluted (neat) suspensions. If lysis was then observed a single plaque was picked off and spread over a Y.D.A. plate already smeared with the strain under test. During propagation of the phage further single plaques were picked to ensure purity of the final phage preparation. The phages thus obtained are referred to as adapted phages. They will be discussed in detail later.

4. STORAGE OF PHAGES

The loss of virulence of a phage for *B. dysenteriae* after storage in the dark at laboratory temperatures was reported by Asheshov (38). Only 0·3% of the phage particles survived after 2 months.

Phages for the lactic streptococci are less adversely affected by storage conditions. Whitehead & Hunter (54) found that Seitz-filtered whey containing these phages could be stored for months in the refrigerator without appreciable loss of titre. Anderson & Meanwell (12) reported that a bacteriophage for a starter strain did not survive 29 days' storage

at 22° C. in whey with an acidity of 0·9%, but when stored in whey of lower acidity, or at 3° C., there was little change over the same period. Yakovlev (7) found that the titre of a phage, causing lysis of a *Str. cremoris* strain, was unimpaired after storage for one month in the dark at 22° C.

The tolerance of these phages to conditions of storage are indicated by (*a*) isolations of phages from old Seitz-filtered whey samples after storage at 1–5° C. for 6 years (p. 175), (*b*) the recovery of active phages from whey powder (p. 177) and from whey concentrate, and (*c*) the recovery of active phage from cheese by Nichols & Wolf (43) after 12 months' storage.

The phage preparations used in this work, after Seitz-filtration, were stored in Y.D.B. or in whey (if prepared in milk) at 1–5° C. Most of the phages retained their titre unchanged under these conditions over long periods, but some, on re-examination, showed a rapid fall in titre. At intervals of about 2 years each phage was rejuvenated on its homologous strain and the new preparation stored as previously.

No attempts have been made to store phages for the lactic streptococci in the dried state, although Swift (31) and Rhodes (32) have successfully stored dried viruses and bacteriophages. There was little difficulty in maintaining the collection of phages in liquid suspension, as indicated above, and only a very few phages have been lost. There was also a serious objection to using the drying equipment and vacuum pump, which were used for drying strains of lactic streptococci, for drying phages, because of the risk of contaminating the strains with phage.

V. ATTEMPTS TO ESTABLISH PHAGE TYPES OF LACTIC STREPTOCOCCI

It is now well known that phages are often selective in their attack on certain strains of organisms within a given species (55). The phenomenon has been used in the classification or subdivision of similar strains of micro-organisms and to identify related strains by their phage 'sensitivity'.

The methods of testing now available and the advances in our knowledge of phage-host variations make possible the differentiation of closely similar types.

Evans (56), working with haemolytic streptococci, suggested that 'the sensitiveness of bacteriophage should be useful in the identification of strains, particularly during epidemics when rapidity of identification is important'. Nichols & Wolf (16) examined the phage-organism affinities of many starter strains and phages isolated from whey, but were unable to divide them into distinct types by susceptibility to phage lysis. Hunter (34) used phages to help in differentiating *Str. cremoris* from *Str. lactis*.

More recently, Whitehead & Hunter (19) have suggested the use as starters of strains of streptococci, unrelated in their phage affinities, to control 'slowness' in cheese-making. They found it difficult to secure more than a few such strains which were active for cheese-making. Hunter (52), describing phage-carrying strains, hinted at further unpublished work on these lines (34, 19).

Babel (57) reported ten bacteriophage types of *Str. lactis* out of fifteen cultures examined. Josse (10) was able to group phages for *Str. lactis* but her numbers, like Babel's, were very small.

Nichols & Wolf (16) found that the choice of the test medium and the establishment of a standard test were difficult, but observed that milk was better than broth for detecting lysis.

Fisk(46) demonstrated lysogenic strains amongst the staphylococci by 'spotting' the potential lysogenic cultures, in high concentration, on to an indicator strain streaked on agar. He used the numerous staphylococcal phages isolated from cultures of *Staph. aureus* to differentiate strains of the organism(58), but he did not enumerate particles or estimate quantitative effects.

Craigie & Yen(59) differentiating types of *B. typhosus* by means of type II Vi phage, suggested a method of critical test-dilution and established the standardization of phage-strain reactions on a reproducible quantitative basis.

Wilson & Atkinson(53) modified Fisk's technique and adapted the method of Craigie & Yen to facilitate the classification of staphylococcal phage types. Williams Smith(60, 61, 62) has pursued this work and has kindly made some of his data available to us.

1. PHAGE TYPING USING STANDARDIZED PHAGE PREPARATIONS ON SOLID MEDIA

The method used is a modification of that described by Wilson & Atkinson(53) and outlined on p. 181. A Y.D.B. culture of each strain to be tested was spread over the surface of a dry agar plate and the critical or test dilution of various phages, i.e. the highest dilution which produced confluent lysis on the homologous strain, was 'spotted' on to the culture with a standard loop. The bottoms of the plates were divided by a glass-writing diamond into sixteen half-inch squares and it was possible, if desired, to 'spot' sixteen different phages on any strain at the same time.

The first sixteen phages examined were selected partly because from previous information they appeared to be dissimilar(16) and partly at random. Many of the strains were not lysed by any of these sixteen test phages and homologous phages for some of the unattacked strains were then used as additional test phages. Certain strains, for which no phage was available at the time, were still not lysed by any of the test phages used and phages already in the collection were adapted to these strains (p. 181). Where the adaptation method was successful the adapted phages were added to the test phages. Of the seventy-eight phages used for typing, forty-five were first propagated on *Str. cremoris* strains, twenty-seven on *Str. lactis* strains, and the remaining six were adapted phages. The phages have been designated according to the strains on which they were first propagated with the exception of the adapted phages which have been numbered *a* 1–*a* 6. There were a few adapted phages of higher designation than *a* 6 but these will be discussed under adaptation, p. 191.

From the details given below, it will be seen that phages propagated originally on either *Str. lactis* or *Str. cremoris* strains were adapted on to both *Str. lactis* and *Str. cremoris* strains.

Adapted phages

No.	Strain on which phage first propagated		Strain on which phage adapted	
a 1	*Str. cremoris*	653/7	*Str. cremoris*	725/33
a 2	*Str. lactis*	X 71	*Str. lactis*	X 14
a 3	*Str. cremoris*	653/7	*Str. lactis*	X 34
a 4	*Str. lactis*	X 71	*Str. lactis*	X 42
a 5	*Str. lactis*	X 71	*Str. cremoris*	893/12
a 6	*Str. lactis*	C 18	*Str. lactis*	C 25

The following notation has been used to show the extent of lysis occurring between the critical dilution of the test phages and the different strains throughout Table 4:

CL	confluent lysis	tr	discrete plaques 1–20
+ +	semi-confluent lysis	Z	zone of incomplete lysis
+	discrete plaques > 20	–	no lysis

Discussion of the results

In all, 375 strains of lactic streptococci were tested in duplicate, against the critical dilutions of seventy-eight phages, involving some 60,000 tests.

The strains were assembled in eleven phage types (Table 4). Other investigations, described later, influenced the subdivision of types I and VIII. In the text the types have been numbered for purposes of discussion, but at this stage of the phage typing such a notation can only be tentative. As more strains of lactic streptococci are examined, new phages prepared to identify untypable strains, and more detailed information on the phage-organism relationships becomes available, it is realized that the types may be subject to alteration or readjustment. The total number of strains allocated to each type is given under each section of the table since the detailed results are only shown for some of the strains. A few of the test phages, too, have been omitted in order to simplify the tables. Thirty-nine strains (10%) were not lysed by any of the test phages and have not been typed.

On examining the available data, at an early stage certain facts were evident:

(1) A few test phages were found to be identical with others.

(2) The placing of all the strains of any one type was rarely determined by a single phage reaction and the types were usually depicted by a 'phage pattern' of a group of indicative phages. The latter were often similar in many respects, but not identical in ability to lyse strains of a type.

(3) Certain phages, e.g. 953/7, *l* 340/1, 457/5 and *a*5, lysed strains of more than one type. Such phage reactions were not taken to indicate any one particular type, except phage 457/5, which was used to identify strains of type IV.

(1) *Differentiation of* Str. cremoris *and* Str. lactis *strains*

Hunter(34) used the evidence obtained from phage sensitivity tests to divide his streptococci into two species, *Str. lactis* and *Str. cremoris*. Although he did not appear to have worked with standardized phage preparations his results in general are confirmed by the present work. The *Str. cremoris* strains appeared to be attacked by phages which were more specific than those attacking *Str. lactis* strains, although there were some exceptions (Table 4). Type VI consists entirely of *Str. cremoris* strains which were lysed by a large number of phages. Types X and XI, composed of *Str. lactis* strains, were lysed by only a few phages which seemed to be type specific.

Whilst either *Str. lactis* or *Str. cremoris* strains only constitute most of the types, type VII contains both species. Type VII*a* and *c* are, with one exception, each composed of *Str. lactis*, but VII*b* consists entirely of *Str. cremoris* strains. A *Str. cremoris* strain has, occasionally, been allocated to a type otherwise composed of *Str. lactis* strains, e.g. type IX*a*, strain 237/7.

Phages originally propagated on *Str. lactis* strains tended to lyse this species only, but, in types III and VIII (Table 5), composed entirely of *Str. cremoris* strains, lysis was recorded with certain phages propagated on *Str. lactis* strains. Only rarely were strains of *Str. lactis* lysed by phages propagated on *Str. cremoris* strains, and never sufficiently strongly for these reactions to be used in typing the strains. These facts also confirmed that the phage reactions amongst *Str. cremoris* strains were more specific than with *Str. lactis* strains, permitting *Str. cremoris* strains to be more readily divided into phage types.

Table 4

Type I*a*

Strains	Phages						
	318*b*/27	314/13	701/2	*ba* 1	*l* 307/2	*a* 7	*a* 8
L307/2	CL	CL	CL	CL	CL	CL	CL
771/4	CL	+	Z	–	–	CL	CL
314/13	–	CL	CL	–	–	CL	CL
198/1	CL	–	Z	–	–	CL	CL
318B/27	CL	–	+ +	–	–	CL	CL
295/9	CL	–	Z	–	–	CL	+ +
198/1/4	CL	+ +	CL	–	CL	CL	CL
295/12	+ +	tr	Z	–	–	CL	Z
198/1/1	tr	–	CL	–	Z	+ +	CL
656/10	–	CL	+ +	–	CL	Z	+ +
295/16	CL	tr	Z	–	–	CL	–
198/1/5	+ +	–	–	–	–	CL	–
198/1/2	–	–	Z	–	–	CL	–
198/1/3	+ +	–	Z	–	–	CL	CL
M6/18	–	CL	Z	–	CL	–	CL

Total no. of strains 36: *Str. cremoris* 36; *Str. lactis* 0.

Type I*b*

Strains	Phages				
	701/2	*ba* 1	953/7	*m* 3/1	*a* 9
701/2	CL	–	CL	–	CL
939/11	+ +	–	CL	Z	CL
953/7	CL	–	CL	Z	CL
P5/2	CL	–	–	–	CL
950/5	Z	–	–	–	–
BA1	–	CL	+ +	–	Z
B22	–	CL	–	–	–
996/12	Z	CL	–	–	–
892/4	Z	–	–	–	–
E8	CL	CL	–	CL	–
Q	CL	–	–	CL	–
R1	CL	–	–	–	–
K7	Z	–	–	Z	–
K8	–	+	–	CL	–
K10	–	–	–	CL	–

Total no. of strains 29: *Str. cremoris* 29; *Str. lactis* 0.

Type II

Strains	Phages		
	l 236/39	324/2	854/1
854/1	–	–	CL
740/2	CL	–	CL
658/4	CL	–	–
L236/39	CL	CL	CL
324/2	–	CL	–
358/10	–	CL	–

Total no. of strains 9: *Str. cremoris* 9; *Str. lactis* 0.

Type III

Strains	Phages		
	457/5	*l* 277/25	*c* 31
L349/1	tr	CL	+
L277/25	–	CL	–
L349/4	tr	CL	+ +
L277/26	–	CL	CL

Total no. of strains 5: *Str. cremoris* 5; *Str. lactis* 0.

Type IV

Strains	Phages 457/5	701/2
457/5	CL	–
L340/32	CL	Z
457/6	CL	Z

Total no. of strains 5: *Str. cremoris* 5; *Str. lactis* 0.

Type V

Strains	Phages *b*3	769/5	701/2	953/7
B/3	CL	tr	tr	CL
654/3	+	tr	Z	+ +
358/2	CL	–	–	+
B/4	+ +	–	–	CL

Total no. of strains 6: *Str. cremoris* 6; *Str. lactis* 0.

Type VI*a*

Strains	Phages 799/11	653/7	810/7	*a*1	953/7	*a*3
725/33	–	–	–	CL	–	–
799/11	CL	–	–	–	–	–
810/9	tr	–	–	–	–	tr
651/8	–	CL	CL	–	–	tr
810/7	–	–	CL	–	–	tr
252/9	+ +	+ +	CL	tr	–	CL
653/7	tr	CL	–	–	CL	+
769/5	CL	CL	+	–	+ +	+ +
651/7	tr	+ +	CL	–	CL	CL
251/10	+ +	CL	CL	–	+ +	CL
319/1	CL	CL	CL	–	CL	CL
295/20	CL	CL	CL	–	CL	CL
252/8	CL	CL	CL	tr	+	CL
267/1	+	+	–	+	CL	+ +

Total no. of strains 37: *Str. cremoris* 37; *Str. lactis* 0.

Type VI*b*

Strains	Phages *s*338/2	339/2	799/11	653/7	810/7	*a*1	953/7	*a*3
650/3	+ +	Z	CL	CL	CL	+	+ +	CL
652/10	Z	Z	CL	CL	CL	–	–	tr
251/8	Z	CL	+	CL	CL	tr	tr	tr
650/1	+ +	+ +	+	CL	CL	+	CL	CL
795/7	Z	–	tr	CL	CL	tr	CL	+
650/6	Z	Z	tr	CL	CL	tr	tr	CL
652/2	+ +	+ +	tr	CL	CL	+	+ +	CL
R652/1	CL	–	tr	+ +	CL	–	+ +	tr
653/6	Z	Z	tr	+ +	CL	–	–	–
652/3	+ +	+ +	tr	+	CL	–	–	CL
R653/1	Z	–	–	–	–	–	tr	tr

Total no. of strains 37: *Str. cremoris* 37; *Str. lactis* 0.

Type VI*c*

Strains	Phages 278/14	*s*338/2	339/2	799/11	821/2	653/7	*a*1	*a*3
Crem J/2	–	Z	Z	+	–	+ +	–	+
RW/4	–	+ +	+ +	+	+ +	CL	+ +	CL
RW/15	+ +	CL	+ +	tr	+ +	CL	tr	CL
RW/18	+	+ +	Z	+	CL	CL	+ +	CL
P8/1	–	–	–	+	–	tr	+	+
821/2	–	–	–	–	CL	–	–	–

Total no. of strains 13: *Str. cremoris* 13; *Str. lactis* 0.

Type VI*d*

Strains	Phages							
	1*p*5	299/2	*s*338/2	339/2	799/11	821/2	653/7	*w*15
262/3	–	–	–	–	–	–	CL	–
339/2	–	CL	–	CL	tr	+ +	CL	–
299/2	–	CL	–	CL	+ +	CL	CL	–
W37	–	–	–	–	–	–	–	Z
166/7	CL	+ +	+ +	CL	tr	CL	CL	CL
L338/27	–	–	CL	–	–	–	–	–
S338/2	–	–	CL	–	–	–	–	–
334/8	+	–	–	–	–	–	–	–
W15	+	tr	–	–	–	–	CL	CL
L311/4	CL	–	–	–	–	–	CL	CL
Y6	CL	–	–	–	–	–	+ +	–
1P5	CL	–	–	–	–	–	tr	–

Total no. of strains 28: *Str. cremoris* 28; *Str. lactis* 0.

Type VI*e*

Strains	Phages			
	*l*324/1	653/7	*w*15	953/7
L324/1	CL	+ +	–	+
L324/2	CL	tr	tr	+
L324/3	CL	–	–	+

Total no. of strains 3: *Str. cremoris* 3; *Str. lactis* 0.

Type VII*a*

Strains	Phages			
	*l*340/1	*s*338/2	339/2	*t*3
X60	CL	Z	Z	CL
X66	+ +	–	Z	CL
X48	+	–	–	+ +
X63	+ +	Z	Z	CL

Total no. of strains 8: *Str. cremoris* 1; *Str. lactis* 7.

Type VII*b*

Strains	Phages		
	701/2	810/7	*t*3
891/6	CL	CL	+ +
891/4	CL	–	CL
891/5	CL	CL	CL

Total no. of strains 5: *Str. cremoris* 5; *Str. lactis* 0.

Type VII*c*

Strains	Phages		
	*t*3	*x*34	*c*30
Y16	CL	tr	+
X34	CL	CL	–
SL2700	CL	–	CL
T3	CL	–	–
C4	+	–	+
C5	CL	–	tr
C6	+	–	–
C29	CL	–	CL

Total no. of strains 12: *Str. cremoris* 1; *Str. lactis* 11.

Type VIII*a*

Strains	Phages								
	144*f*	167/11	306/14	620/4	*l*326/2	*x*17	*l*459	*a*10	*a*12
B/7	CL	CL	–	–	CL	CL	CL	CL	CL
L326/2	CL	CL	++	CL	CL	+	CL	CL	CL
P4/1	CL	++	–	–	–	–	+	CL	CL
174/11	+	CL	CL	CL	–	tr	CL	CL	CL
B/25	CL	tr	–	–	–	CL	–	tr	CL
794/1	–	CL	+	CL	–	–	CL	CL	CL
306/14	–	–	CL	–	–	–	–	CL	Z
172/3	–	CL	CL	Z	–	–	–	CL	CL
209/6	–	++	–	–	–	–	CL	CL	Z
167/11	–	CL	–	–	–	–	–	Z	++
156/5	CL	–	–	–	–	CL	++	–	CL
144F	CL	CL	–	CL	CL	CL	–	–	CL
156/2	++	–	–	–	–	CL	++	–	CL

Total no. of strains 28: *Str. cremoris* 28; *Str. lactis* 0.

Type VIII*b*

Strains	Phages	
	620/4	*l*459
620/4	CL	–
L459	–	CL
340/1	CL	–
ML/1	–	CL

Total no. of strains 5: *Str. cremoris* 5; *Str. lactis* 0.

Type IX*a*

Strains	Phages						
	*n*35	*x*17	*x*71	*m*2*s*1	*t*2	*a*2	*a*4
N35	CL	CL	–	–	–	–	–
237/7	CL	CL	CL	CL	CL	CL	CL
X14	–	CL	–	–	–	CL	CL
X17	–	CL	–	–	–	CL	CL

Total no. of strains 6: *Str. cremoris* 1; *Str. lactis* 5.

Type IX*b*

Strains	Phages						
	*x*71	*m*2*s*1	*m*3/1	*t*2	*a*2	*a*4	*b*20
M3/1	–	CL	CL	–	CL	Z	CL
M2S1	CL	CL	CL	–	CL	CL	–
X40	tr	tr	–	–	CL	CL	CL
M16/4	++	CL	–	CL	–	–	CL
N40	+	–	CL	++	CL	CL	CL
T2	–	–	–	CL	–	–	–
X62	CL	–	–	CL	CL	CL	CL
T1	–	–	–	CL	–	–	–
X71	CL	–	–	CL	–	–	–
C33	–	–	–	–	CL	CL	CL
X43	–	–	–	–	CL	CL	CL
C17	–	–	–	–	–	–	CL

Total no. of strains 50: *Str. cremoris* 0; *Str. lactis* 50.

Type X

Stains	Phages		
	*c*8	*t*8	*c*31
C8	CL	CL	CL
C31	CL	CL	CL
T8	CL	CL	–
T4	CL	–	–

Total no. of strains 12: *Str. cremoris* 0; *Str. lactis* 12.

Type XI

Strains	Phages
	*bw*2
BW2	CL
BW8	CL

Total no. of strains 2: *Str. cremoris* 0; *Str. lactis* 2.

The *Str. lactis* strains, with a few exceptions, fell into three phage types (IX, X and XI). Types X and XI embraced only a few strains and the reactions of the indicative phages were confined to the strains of the respective types. Type IX*b*, however, consists of fifty strains, lysed to a greater or lesser extent by several phages, all originally propagated on *Str. lactis* strains. The indicative phages varied in many characteristics, for example, plaque size and loss of titre on storage, suggesting the possibility of further subdivision.

(ii) *Typing by a 'phage pattern'*

Wilson & Atkinson (53), typing strains of staphylococci, noted that some types contained strains which were lysed by one phage only, others included strains lysed by more than one test filtrate and they suggested that the former might prove to be susceptible to lysis by other phages isolated in the future. One of the eleven types suggested for the lactic streptococci is composed of strains (two) lysed by only one phage (type XI) but the isolation of further indicative phages is not precluded.

Type X is an example of a collection of strains whose phage-organism relationships were relatively simple. Phage *c*8 completely lysed all strains of the type and this reaction appears to be determinative. Some of the constituent strains were also lysed by two other test phages, *c*31 and *t*8, but because phage *c*8 lysed these and no other strains in the collection, the strains were placed together to form one phage type.

The phage-organism relationships of the large number of strains in type VIII*a* are more complex, and it is not so easy to justify the inclusion of some of them. If the reactions of the adapted phage *a*12 (discussed later) are ignored, phage 167/11 lysed, at least partially, more of the strains placed in type VIII*a* than any other phage. If only one phage were to be selected for identification of the type, 167/11 seems to be the best choice, but three strains 306/14, 156/5, 156/2 would have to be excluded. Many of the strains lysed by phage 167/11 were lysed, in addition, by other phages, viz. 144*f*, 306/14 and *l*326/2, none of which reacted with any strains other than those of type VIII*a*. A further phage, *x*17, whilst lysing many strains of type VIII*a* also lysed strains allocated to other types (IX*a*): these latter reactions, because they were not confined to one type, were ignored in identifying the strains of type VIII. The three strains which were unattacked by critical dilutions of phage 167/11 were lysed by one or other of the phages whose reactions were otherwise confined to strains of type VIII*a*. This fact was regarded as sufficient reason for including these three strains in type VIII*a*.

From these examples it will be seen that the phage types have been identified, not necessarily by the reaction of a single phage but by the lysis of one or more of a selection of phages, or a 'phage pattern'.

(iii) *Diversity of phages forming a 'phage pattern'*

Type VI contains the largest number of strains (118) and is identified by a wider range of phage reactions than any other type suggested, its constituent strains being exclusively *Str. cremoris*. The phages regarded as indicative for this type can roughly be divided, by certain characteristics, into three groups:

(*a*) 799/11, 653/7, *a*1 and *a*3. High titre lysates could readily be prepared in Y.D.B. or milk and all lysates produced very large plaques.

(*b*) *s*338/2, 339/2, 810/7 and 821/2. It was difficult to obtain high titre lysates either in Y.D.B. or milk and often the phages did not show lysis by clearing when grown in Y.D.B. The phage titre often decreased during storage and plaques were usually small.

(*c*) 1 *p* 5, *w* 15 and 299/2. Lysis in Y.D.B. or milk was evident, but plaques were smaller than in (*a*).

Arrangement of strains which were obviously of type VI was difficult. Strains of type VI*a* were grouped together by their susceptibility to the phages detailed under (*a*) and phage 810/7. Type VI*b* strains, in addition to being lysed by phages of (*a*) were lysed by three phages from (*b*), viz. *s*338/2, 810/7 and 339/2. Strains of type VI*c* were lysed by phages indicative for types VI*a* and VI*b* and by the remaining phage, 821/2, mentioned under (*b*). Type VI*d* strains were lysed by phages from (*c*) and by *s*338/2 and 339/2 from (*b*), but were less susceptible to lysis by phages of (*a*). The three strains of type VI*e* were lysed by phages under (*a*) (see also Table 5), and moreover by a test phage, *l*324/1, not reacting with any other strain of type VI.

The typing of some of the strains, particularly in VI*d*, has been tentative, and further evidence was still required to confirm their position in that type.

(iv) *Adapted phages in the test collection*

The range of lysis of the adapted test phages was compared with that of the respective original test phages from which they were adapted. Phage *a*1, adapted from phage 653/7, lysed in critical dilution fewer strains (type VI) than the original phage, and failed to react with strain 653/7 in that dilution. Phage *a*3, also adapted from phage 653/7, lysed, on the whole, the same strains as the original phage. Whilst lysis by phages *a*2 and *a*4, adapted from phage *x*71, was confined to strains of type IX, more strains were lysed by these phages than by phage *x*71. Neither adapted phage in critical dilution lysed strain X71. A further adapted phage, *a*5, of similar origin, did not lyse strain X 71 or the strains of type IX. The phage-organism reactions of phage *a*5 were not limited to any one type and it was not regarded as a determinative phage for any type. There did not, from these results, appear to be any general rule to indicate the range of lytic activity of adapted phages compared with that of the original phage.

(v) *Strains from a starter and substrains from a strain*

As already shown by Nichols & Wolf(16), not all strains isolated from one starter necessarily showed the same phage-organism relationships, and in the present work such strains did not always belong to the same type. For example, strains 295/9, 295/12 and

295/16 fell into type I*a* whilst strain 295/20, also isolated from the same starter (295), was placed in type VI*a*. Substrains, from a single strain, belonged to the same type. The substrains of strain 198/1 (198/1/1–198/1/5) had been isolated some years before testing. Not all the substrains were identical in susceptibility to lysis by the test phages, but they all reacted with the same 'phage pattern' (type I*a*).

(vi) *'Zone' reactions*

Craigie & Yen(59), discussing the difficulties of interpreting phage tests, noted the occurrence of some strains, the growth of which was not completely inhibited by the homologous phage. The cultures showed an atypical translucency in the area occupied by the phage. Evans(44) reported 'zoning' with phages from the haemolytic streptococci. She noted that when a certain phage-organism combination showed the 'zone' phenomenon it recurred under the same test conditions. Where 'zoning' was observed with the lactic streptococci (indicated in Table 4 by Z) there was clearly some phage reaction, but on the area which would normally have been completely lysed there was a thin mat of growth. This occurred frequently with some of the test phages, e.g. 701/2, *m*3/1, *s*338/2, 339/2 and a number of phages propagated on recently isolated *Str. lactis* strains. It may be significant that these phages, with the exception of phage 339/2, were applied undiluted (neat). The critical dilution of phage 339/2 was only 10^{-1}. Craigie & Yen(59) found that certain strains which showed a relatively early development of growth, resistant to the phage applied, yielded 'weaker phage showing a lower degree of specificity'. In preparing test phages for the lactic streptococci difficulties were encountered in obtaining high-titre lysates for some strains, particularly those which rapidly developed resistant secondary strains. The period of visible lysis was either very limited or absent in Y.D.B. cultures. The phages causing 'zoning' on the plates were of this kind and, had it been possible to 'build up' a phage of higher titre for some of these strains, the phage reactions might have been more specific and 'zone' reactions might have been eliminated. Although the titre may influence the specificity of a phage other factors are involved, since phage *l*340/1, the least specific of the test phages propagated on *Str. cremoris* strains, was applied in higher dilution than the majority of the test phages.

(vii) *Strains not included in the types*

Of the thirty-nine strains not typed, twelve showed slight reactions with one or other of the test phages, but insufficient to justify their placing. The remaining twenty-seven were not lysed by any of the test phages. For some of these strains phages have been isolated but, owing to the minute plaque size, purification was questionable and they were excluded from the test collection. Lysis was observed for some of the other strains in milk culture (p. 176), but a purified phage suspension was not obtained, as phages could not be recovered from the Seitz-filtrates. With adjustments of the present technique it should be possible to isolate phages for these strains also.

2. ADAPTATION—A SEARCH FOR A PHAGE CAPABLE OF LYSING ALL THE STRAINS OF ONE TYPE

In the suggested classification for the lactic streptococci the type usually depended for identification upon a differential 'phage pattern'. If one phage could be obtained, capable of lysing every strain in a type, not only would it simplify the typing of new strains, but it

would also confirm the doubtful position of some strains and perhaps indicate where certain of the types should be subdivided.

Many records exist of phage variation and adaptation, and it is accepted that when a high-titre phage is plated with a resistant bacterial mutant some plaques often develop on incubation. This phenomenon has been exploited by Wilson & Atkinson (53) and Williams Smith (60, 61, 62) for the staphylococci and in this work on the lactic streptococci, to obtain phages for untypable strains of organisms. Whitehead & Hunter (63) reported that they were unable to adapt a phage for a heterologous organism on to a strain of *Str. cremoris* in a liquid medium.

Another property of adapted phages has been used for typing typhoid and paratyphoid strains. Craigie & Yen (59) obtained adapted phages from a particular anti-Vi phage which were highly specific for the strain on which they had been propagated. These new phages lysed strains which were related epidemiologically and divided the Vi strains of *B. typhosus* into several types. Similarly, Felix & Callow (64) later evolved a scheme of phage typing for the Vi strains of paratyphoid B.

Methods of investigation

At first, efforts were made to adapt phages to replace the 'phage pattern' already used to establish types I and VIII and later the work was extended to type VI. The adapted phages were prepared by the general method described earlier (p. 181). For example, one of the diagnostic phages for type VIII, 167/11, failed to lyse all the strains tentatively placed in this type when 'spotted' in critical dilution on to them. It was then 'spotted' neat, i.e. without dilution, on to all the previously non-reacting strains. When any plaques were obtained, attempts were made to 'build up' and purify a phage on the strains previously unattacked by phage 167/11. This phage was adapted on to strain 156/5 and the adapted form of the phage obtained (*a* 12) was 'spotted' in critical dilution on to all the strains previously classified in type VIII. It was hoped that the adapted form of such a phage as 167/11 might react with the strains lysed by phage 167/11 and, in addition, the strains of that type resistant to lysis by the original phage, and so confirm or clarify the typing.

Many unsuccessful attempts were made to adapt some of the phages on to one or more of the strains. Five adapted phages (see below) were finally used to assist the classification of types I and VIII, but these were selected from some nine adapted phages prepared for type I and eight prepared for type VIII after they had been tested against all the strains previously classified in these types.

No.	Strain on which phage first propagated	Strain to which phage adapted
a 7	*Str. cremoris* 318*b*/27	*Str. cremoris* 303/4
a 8	*Str. cremoris* 314/13	*Str. cremoris* 198/1
a 9	*Str. cremoris* 953/7	*Str. cremoris* P5/1
a 10	*Str. cremoris* 167/11	*Str. cremoris* 306/14
a 12	*Str. cremoris* 167/11	*Str. cremoris* 156/5

Discussion of results

(1) No single adapted phage has been found to lyse all the strains classed as one type and so replace the 'phage pattern' on which the type was created. Some adapted phages completely lysed most of the strains, for example, phage *a* 7 only failed to react with one strain M 6/18 in type I *a*, and when 'spotted' on to the remaining thirty-five strains in this

type resulted in confluent lysis with twenty-seven. Although phage *a*10, adapted from phage 167/11, lysed strain 306/14 with which phage 167/11 did not react in critical dilution, it only reacted with nineteen out of twenty-eight strains in type VIII*a*, i.e. the same number but not exactly the same strains as the original phage 167/11.

(2) The adapted phages indicated the subdivision of types I and VIII. In type I*a* the adapted phages *a*7 and *a*8 reacted with most of the strains but neither reacted with any of the strains later classed as type I*b*. Similarly, the adapted phage *a*9 helped to separate I*b* from I*a*. In the original classification of type VIII five strains, now identified as VIII*b*, were included. There was justification for including them on an equal basis with the three strains 156/2, 156/5 and 306/14 not lysed by phage 167/11, since they were each lysed by one phage of the indicative 'phage pattern' for type VIII. The position of strains 156/2, 156/5 and 306/14 was later strengthened by their susceptibility to adapted phages *a*10 and *a*12, but the resistance of the remaining five strains to these adapted phages was regarded as additional evidence for creating subtype VIII*b*.

(3) The clarification of type VI (118 strains) was attempted by using adapted phages. Difficulties were encountered in 'building up' adapted phages of high concentration. It became obvious too, that it would be difficult to adapt one or two phages to react with large numbers of strains.

(4) During the work with strains in type VI, a search showed that it would be hard to find a phage for the lactic streptococci similar to that obtained by Craigie & Yen (59) or by Felix & Callow (64) and adapted by them on different strains in turn, until a number of distinct subtypes had been identified. If a phage, possessing this adaptability, had been encountered and could have been applied to classify these organisms the phage typing would then have been readily reproducible elsewhere by other workers when supplied with the same phage.

Typing strains by 'phage pattern', as first developed on a large scale by the workers on staphylococci and now suggested for the lactic streptococci, has many disadvantages, not the least of which are, the maintenance of the diagnostic phage collection and homologous strains and the distribution of the test phages and strains to other workers.

(5) Craigie & Yen subdivided an antigenic type of the *Salmonella*, which had already been clearly identified by serological methods, into further subtypes by phage. There are several serological types amongst the strains of group N streptococci (65) but the subdivision is not complete and there is no indication where to start applying a method of phage typing dependent on adaptation from one phage only. Serological typing of the staphylococci has not yet, and may never be, developed sufficiently far to simplify the phage-typing methods of identification at present used for *Staph. aureus*. No forecast can be made from the results with staphylococci as to the extent to which the establishment of serological types of group N streptococci might assist in the phage typing of the lactic streptococci.

3. THE SIGNIFICANCE OF 'PHAGE-RESISTANT CARRIER' STRAINS IN PHAGE TYPING

Although lysogenic strains have been detected in many groups of organisms, no method has been described for detection of lysogenic strains amongst the lactic streptococci, and the identification of a lysogenic strain of *Str. lactis* or *Str. cremoris* has not yet been reported in the literature. Hunter (52) first noted the occurrence of a strain of *Str. cremoris* existing in a symbiotic state with a phage, but unlike true lysogenic strains, on plating and repicking

the phage could no longer be detected on the cultures obtained. Similarly, the resistant secondary culture arising after lysis of a strain was found to be capable of carrying the phage which stimulated its appearance. On plating and repicking isolated colonies, the substrains obtained no longer carried the phage, although retaining their resistance to lysis. He noted that phage was not carried by all resistant secondary cultures and that during their repeated subculture the phage was lost by the process of dilution. Those carrying the phage often retained the property for long periods of serial subculture. It could not be predicted for any given resistant strain how long the phage would persist.

How commonly the phenomenon of phage carrying by strains of *Str. lactis* and *Str. cremoris* occurs in the field is not known, since all strains in the present collection were purified by plating, thus eliminating carried phage during purification. For the same reasons phage carrying has not been detected amongst laboratory cultures. The phage-carrying phenomenon, as described by Hunter, has been confirmed by examination of resistant secondary strains, before and after purification by plating, for the presence of the phage initially added.

Williams Smith (60), investigating typing of staphylococci by bacteriophage, made observations on lysogenic strains of staphylococci which might have a parallel in phage-carrying strains of lactic streptococci. He stated that acquired phage resistance was an important factor in the phage typing of strain of staphylococci. The classification into a larger number of types than would otherwise have been possible was an advantage in epidemiological studies but, otherwise, it was a complicating factor since strains, identical except for acquired phage resistance, might be classified as different types. When resistant strains were lysogenic the character could be utilized to identify other strains differing only in acquired phage resistance. He noted that in the type 42 D the non-lysogenic strains were fully susceptible to all the phage filtrates which he had prepared for sub-dividing the group, but the lysogenic strains were only susceptible to some of them. In a further paper (61) Williams Smith showed that some staphylococcal strains had been classified by Wilson & Atkinson (53) into different types or subtypes, by their susceptibility to several test phages, because some of them had been previously infected with one or more different test phages for the type. The phage resistance thus acquired accounted for their resistance to some of the test phages diagnostic for the type.

The suggested typing for the lactic streptococci depended upon the reaction of the strains to several phages (the 'phage pattern') because, like the staphylococci, strains were rarely lysed by all the phages indicative for the type. It may be that strains of lactic streptococci of the same type, resistant to one or more of the indicative phages, are not lysed because, through previous lysis, they have acquired resistance to those phages in the field. As the occurrence of lysogenic strains of *Str. cremoris* or *Str. lactis* has not yet been demonstrated it is not possible to confirm this hypothesis by methods used for the staphylococci.

In view of the work of Hunter & Williams Smith it was possible that phage-carrying properties might be used to elucidate some of the phage-typing results. If it could be shown that the phages indicative for one type were not carried by strains of other types and resistant strains in a type could carry the phages, determinative for the type, to which they were resistant, i.e. phage carrying was type specific, this phenomenon could be used to confirm the phage typing. The ability or failure of strains of lactic streptococci to carry a phage could be used to strengthen the position of strains already tentatively placed in

a type by the results of cross-phaging tests (Table 4) and to assist in the possible subdivision of some types.

Methods of investigation

The strains were examined, usually in litmus milk, for capacity to carry a particular phage. Two drops of the filtered phage suspension were added to 10 ml. of litmus milk previously inoculated with the strain under test. The mixture was transferred daily for at least seven serial subcultures. Since the dilution was approximately 10^{-2} daily, by the seventh day the phage originally added was eliminated by dilution if not carried on the strain under test. At the eighth subculture two drops of the culture grown from the seventh transfer were added to 10 ml. of litmus milk seeded with the homologous strain for the phage inoculated initially. The presence or absence of phage was determined on the following day by plating dilutions of the mixed culture with the homologous strain on Y.D.A. The incubation temperature throughout was 30° C.

The strains in types VIII*a* and *b*, VI*c* and *d* and type I*a* were tested for ability to carry different phages which were indicative of the respective types. Each of a number of strains was examined for capacity to carry phages specific for types other than the one to which it belonged. The details of the phage-carrying experiments are given under the discussion of results and summarized in Table 6.

When testing resistant strains of type VIII for capacity to carry phages for that type several of the cultures grew slowly in milk, having lost the power to produce acid rapidly before the best method of storage had been adopted. In order that growth might be readily observed 24 hr. after subculture instead of after several days, Y.D.B. was substituted for litmus milk as the culture medium.

Interpretation of results

When a resistant secondary culture arises as a result of phage lysis, it has been shown for the staphylococci (60) that the resistant strain may or may not 'carry' the causal phage. In the latter instance there is also the possibility that the phage may have been present but not detected by the method employed. In the interpretation of phage-carrying observations for the lactic streptococci a positive result indicated the capacity of the culture to 'carry' the phage, but a negative result might merely indicate faults in technique. A positive result was sometimes obtained for a strain previously negative when the phage was again added and the same methods for detection used, but such a false negative result had already been noted by Hunter. He reported that similar phage-culture mixtures when tested for phage carrying might give different results.

The method adopted here for detection of phage carriers would be unsuitable for identifying lysogenic strains of the staphylococci, but it was adequate for the purpose; final plaque reading either indicated a high titre of phage in the mixture or completely failed to demonstrate the presence of phage and few doubtful readings were encountered.

Discussion of results

(i) *Capacity of strains for carrying phages not indicative of the type*

Some phages capable of lysing strains of lactic streptococci were not type specific in their reactions. Possibly many of the strains already assigned to types, but not lysed by these non-specific phages, might be capable of carrying them. Should this occur it would

not be regarded as determinative. If, however, phages lysing strains of one type only were carried by strains of a different type no useful purpose, in respect of the phage typing of strains, would be served by further investigation of 'phage-resistant carrier' strains.

Phage *x*17, a phage lysing strains of more than one type (types VIII*a* and IX*a*), was added to fourteen strains from types not lysed by critical dilutions of that phage and to all strains of types VIII and IX*a*. The strains were tested for ability to carry the phage as already detailed (p. 196). Although the phage reactions were not confined to one type it was not possible to demonstrate the carriage of phage *x*17 by any of the fourteen strains selected from types other than VIII*a* or IX*a*. Strains of types VIII*a* and IX*a*, susceptible to lysis by phage *x*17, gave resistant secondary cultures which carried phage *x*17. This phage must also have multiplied on some of the resistant strains of type VIII*a*, e.g. P4/1 and 306/14, since positive results were obtained.

When a further non-specific phage 457/5 was added to a collection of fifteen strains it was found that 'carried' phage could be detected on some of these strains which had been selected from a diversity of types.

One hundred and two strains were each tested for ability to carry a phage specific for a type other than the one to which it had been assigned. The phages used were also added to their respective homologous strains and the resistant secondary cultures which arose were tested for ability to carry the phage initially added. A selection of the results is given in Table 6*a*. With the exception of phage *l* 459, each phage was carried by the resistant secondary culture arising after lysis of the homologous strain. Of the strains tested, one hundred, i.e. 98%, failed to carry phages diagnostic for heterologous types. The results encouraged further experiments.

(ii) *Capacity of resistant strains in a type to carry phages indicative of the type*

When a phage type contained a few strains lysed by a small 'phage pattern' some of the constituent strains were susceptible to lysis by every phage in the 'pattern'. If the 'phage pattern' consisted of a number of phages a strain was only rarely lysed by every phage indicative of the type, and in types VIII*a* and *b* and types VI*c* and *d* most of the strains were resistant to one or more of the phages indicative of the respective types. Each strain, which was resistant to certain phages of the 'phage pattern', was tested for ability to carry those phages. Phages were also added to many of the susceptible strains to test further the capacity of cultures, which had acquired resistance under experimental conditions, to carry the phage initially applied (Table 6*b* and *c*).

Types VIII a and b. All the strains susceptible to lysis by phages 144*f* and 167/11 developed resistant secondary cultures which carried these phages when added. With phages *l*326/2 and *l*459 not all such resistant secondary cultures were shown to carry the phage applied. It was possible, therefore, that some of the resistant strains in the type, which had acquired resistance in the field, might also fail to carrry these two phages.

The subdivision of type VIII into *a* and *b* was confirmed by the failure of all strains in type VIII*b* to carry phage 167/11 whilst those strains in type VIII*a* not lysed by phage 167/11 (306/14, 156/2 and 156/5) were able to do so. The ability of strain 306/14, the most doubtfully placed of all the strains in the type, to carry phage *l*459 further confirmed the inclusion of this strain in type VIII*a*.

Types VI c and d. Phage 339/2, when added to susceptible strains, gave rise to resistant secondary cultures, all of which carried the phage. The results with other phages for the

Table 6*a*. *Incapacity of strains to carry phages diagnostic for other types*

Type	Strain	Phages and types for which diagnostic: Ia 318*b*/27	V *b*/3	III *l*277/25	VII *t*3	VIII 144*f*	VIII 167/11	VIII *l*459	VIII 236/9	VIII *l*326/2
I*a*	318B/27	*	–	–	–	–	–	–	–	–
V	B/3	–	*	–	–	–	–	–	–	–
III	L277/25	–	–	*	–	–	–	–	–	–
VII	T3	–	–	–	*	–	–	–	–	–
VI*d*	1P5					–	–	–	–	–
VI*a*	267/1					–	–	–	–	–
VI*c*	RW13					–	–	–	–	–
IX*b*	X71					–	–	–	–	–
I*a*	L307/2					–	–	–	–	–
IX*b*	M2S1					–	–	–	–	–
II	L236/39	–				–	–	–	–	–
IX*b*	C1					–	–	–	–	–
X	C8					–	–	–	–	–
VI*c*	L324/1					–	–	–	*	*

*=Phage carried when resistant. –=No phage carried.

Table 6*b*. *Phage lysis* (1) *and phage-carrying reactions* (2) *in types VIII a and b*

Strains	144*f* (1)	144*f* (2)	167/11 (1)	167/11 (2)	236/9 (1)	236/9 (2)	*l*326/2 (1)	*l*326/2 (2)	*l*459 (1)	*l*459 (2)	*x*17 (1)	*x*17 (2)
B/7	CL	*	CL	*	–	*	CL	*	CL	*	CL	*
L311/10	CL	*	CL	*	+	*	CL	*	CL	*	CL	*
L326/2	CL	*	CL	*	+ +	*	CL	*	CL	*	+	*
L326/20	CL	*	CL	*	+	*	CL	*	CL	*	CL	*
P4/1	CL	*	tr	*	–	*	.	*	+	*	–	*
144F/1	CL	*	CL	*	–	*	CL	*	CL	*	CL	*
158/2	CL	*	CL	*	–	*	CL	*	+	*	Z	*
174/11	+	*	CL	*	CL		–	*	CL	–	tr	*
B/25	CL	*	tr	*	–	–	–	*	–	*	CL	*
236/9	–	*	CL	*	CL	*	–	*	CL	*	–	*
L277/1	–	*	CL	*	+ +	*	–	–	CL	*	–	–
794/1	–	–	CL	*	+ +		tr	*	CL	–	–	*
236/6	–	*	+	*	–	*	–	*	+ +	–	–	*
306/9	–	*	CL	*	tr	–	–	*	+ +	–	–	*
306/14	–	–	–	*	–		–	–	–	*	–	*
172/3	–	*	CL	*	CL	*	–	–	–	*	–	*
209/6	–	–	+ +	*	tr	–	–	*	CL	–	–	*
167/11	–	*	CL	*	–	*	–	*	–	*	–	*
156/1	CL	*	CL	*	–	*	–	*	–	*	CL	*
156/5	CL	*	–	*	–	*	–	*	+ +	*	CL	*
144F/3	CL	*	+ +	*	–	*	–	*	tr	*	CL	*
P4/2	CL	*	CL	*	–	*	–	*	CL	*	–	*
144F	CL	*	CL	*	–	*	CL	–	–	*	CL	*
156/2	+ +	*	–	*	–	*	–	*	+ +	–	CL	*
620/1	–	–	–	–	–	–	–	–	–	*	–	–
620/4	–	–	–	–	–	–	–	–	–	–	–	–
L459	–	*	–	–	–	–	–	–	CL	–	–	*
340/1	–	*	–	–	–	–	–	–	–	–	–	–
ML/1	–	–	–	–	–	–	–	–	CL	–	–	–

*=Phage carried when resistant.
–=No phage carried.
Notation for degrees of phage lysis, p. 183.

Table 6*c*. *Phage lysis* (1) *and phage-carrying reactions* (2) *in types VI c and d*

	Phages											
	1*p*5		299/2		339/2		653/7		*w*15		262/3	
Strains	(1)	(2)	(1)	(2)	(1)	(2)	(1)	(2)	(1)	(2)	(1)	(2)
Type VI*c*												
Crem J/2	–	–	–	–	Z	*	++	*	–	–	–	–
Crem J/1	–	*	–	*	CL	*	tr	*	–	*	–	–
RW/6	–	–	–	–	Z	–	CL	–	–	*	–	–
RW/12	–	*	–	*	CL	*	CL	*	–	*	–	*
RW/15	–	–	–	*	++	*	CL	*	–	*	–	*
RW/18	–	*	–	*	Z	*	CL	*	–	*	–	–
HP	–	*	–	–	–	–	+	*	–	*	–	–
821/2	–	–	–	*	–	*	–	*	–	*	–	–
Type VI*d*												
262/3	–	–	–	–	–	*	CL	*	–	–	CL	–
368/1	–	*	++	*	CL	*	CL	*	–	*	+	*
339/1	–	*	++	*	CL	*	CL	*	–	*	tr	*
299/2	–	*	CL	*	CL	*	CL	*	–	*	–	–
278/14	–	–	CL	–	CL	*	CL	–	–	*	–	*
166/8	–	*	–	*	++	*	CL	*	–	*	–	–
W37	–	*	–	*	–	*	–	*	Z	*	+	*
166/7	CL	–	++	–	CL	*	CL	*	CL	–	++	–
216/9	+	–	–	–	–	–	CL	*	CL	–	–	–
L338/5	–	*	–	–	–	–	–	*	Z	–	–	–
L338/27	–	–	–	–	–	–	–	–	–	*	–	–
S338/2	–	*	–	–	–	–	–	–	–	*	–	–
S338/10	++	*	–	–	–	–	–	*	CL	*	–	–
334/8	++	*	–	*	–	*	–	–	–	–	–	–
W15	++	*	tr	*	–	*	CL	*	CL	*	–	*
W18	tr	*	–	–	–	*	tr	–	CL	*	–	*
L314/4	CL	*	–	*	–	–	CL	*	CL	*	–	–
L311/4	CL	*	–	–	–	–	CL	–	CL	–	–	–
798/2	CL	*	–	–	–	–	CL	–	Z	–	–	–
Y1	CL	*	–	–	–	*	–	–	–	*	–	–
M4/2	CL	*	–	–	–	*	+	–	–	–	–	–
1P5	CL	*	–	*	–	–	tr	–	–	–	–	–

* = Phage carried when resistant. – = No phage carried.
Notation for degrees of phage lysis, p. 183.

type were variable and in some of the resistant secondary cultures, similarly obtained, the phage initially applied could not be detected after the usual daily transfers. It was likely that some of the resistant cultures in the type might also fail to carry the added phages.

Phage 653/7, which lysed strains throughout type VI, lysed most of the strains and was also carried by some of the resistant strains in each subtype. Failure to carry the phage together with resistance to lysis, was found in only four of the thirty-four strains examined.

The property of strains in type VI*c* to carry phages 1 *p* 5, 299/2, *w*15 and 262/3 was surprising since none of these phages lysed any of the strains in that subtype. Provided that the phage-carrying results have been interpreted correctly, types VI*c* and VI*d* appeared to be more closely related than results from the cross-phaging tests on agar had indicated. Strain 821/2 was placed in type VI*c*, since it was lysed only by its homologous phage 821/2 which also lysed other strains of the type. Its capacity to carry four phages also carried by the other strains in the type, confirmed the orginal placing. Similarly, strain HP, lysed by phage 653/7 only, was found to carry three of the six phages applied. Identification of type VI*d* strains was more difficult and the placing of certain strains, e.g. 262/3, W 37, L 338/5, L 338/27, S 338/2, S 338/10 and 334/8, has been tentative. Each of these strains was found to carry at least one of the phages applied, and even if this could be regarded as confirmatory evidence in favour of their assignment to type VI*d* their position is still uncertain.

Type Ia. With the exception of strain 822/1, all the strains not lysed by the critical dilution of phage 318*b*/27 were able to carry the phage. Similarly, M 6/18, the only strain in Type I*a* not lysed by adapted phage *a*7 was able to carry that phage. The position of the most doubtfully placed strain in the type (M 6/18) was thus confirmed.

4. PHAGE TYPING OF THE LACTIC STREPTOCOCCI, PHAGE ADAPTATION AND THE PHAGE-CARRYING PHENOMENON. THE SIGNIFICANCE OF THESE FACTORS IN THE SELECTION OF STARTER STRAINS

It has been shown that strains from a wide collection of lactic streptococci could be divided into types by their susceptibility to lysis by standard dilutions of phage. This method provides the best means available, at present, for the differentiation of any one strain from another within the species *Str. cremoris* or *Str. lactis*. The types suggested can be extended or modified to include further strains and phages isolated in the future. Owing to the occurrence of strains which may have acquired resistance to a phage under field conditions, the method is of limited value as a means of establishing the identity of a strain. However, in conjunction with data of the capacity of such resistant strains to carry added phages, it may be used to indicate strains which are similar except for acquired phage resistance.

For the routine typing of potential starter strains the 'spotting' of standard phage dilutions on to the strain on agar appears to give a reasonably sound indication of the type to which a strain belongs. The determination of the phage-carrying capacity of any strain, not placed with certainty by the first method, should help to place the strain more definitely. Additional information on the phage-carrying capacity of strains from types, not yet examined for that property, would help. The information on the phage-organism relationships of the strain, acquired whilst typing the strain, can then be further utilized to select unrelated strains for starter.

Until a phage yielding other phages, capable of lysing all strains of lactic streptococci, is found the method of phage typing developed by other workers (59, 64), for different groups of organisms, cannot be applied to the lactic streptococci.

During the cheese-making process phage, capable of lysing the starter strains in current use, can usually be detected in the whey. Reports from New Zealand show that when a stock culture had not been used in a factory for several months, the specific phage rarely failed to reappear in the whey during the first 2 days after the reintroduction of the stock culture as starter. Furthermore, where a starter had been used in a cheese factory for a long period there was invariably at least a low concentration of phage in the whey, probably due to airborne contamination of the milk whilst in the open vats. Rotational use of a collection of strains, unrelated in phage susceptibility, was introduced to prevent a dangerous 'build up' of this phage concentration (19). In this country, at an earlier date, similar methods to prevent phage concentration from reaching a dangerous level had been adopted which reduced the importance of phage contamination of cheese milk from factory equipment and from the air.

The significance of the phage-carrying phenomenon cannot be ignored in selecting the component strains for a starter rotation. Applying the phage-typing data two strains might be selected from the same type which are not lysed by the same phages and which would appear to be unrelated by cross-phaging tests alone, e.g. strains HP and 821/2 (Table 4, type VI*c* and Table 6*c*). Strain HP was lysed by phage 653/7 only and strain

821/2 by phage 821/2 only, but as strain 821/2 may carry phage 653/7, the use of the strain HP and 821/2 on consecutive days would defeat the object of rotational use of strains. Phage 653/7, present in the factory when strain HP is in use in the vat, would normally multiply on strain HP. On introducing strains 821/2 the following day, phage 653/7 may be carried on that strain without apparent lysis but with possible further increase in concentration of the phage. Little advantage in the control of phage 'build up' would therefore be gained from using these two apparently different strains in the same starter rotation.

Since some of the phages indicative of a type can be adapted to resistant strains of the same type it is not impossible that such adaptation might occur in cheese factories. On these grounds too, it would be advisable to select strains, for rotational use as starter from different phage types rather than strains which appear to be dissimilar only by virtue of susceptibility to phage lysis.

Classification of starter strains by phage typing can obviously not be attempted in the factory. The establishment of a reference laboratory for the lactic streptococci, in order to typé potential starter strains, would be of assistance to the cheese-making industry. A few of the larger cheese manufacturers have already attempted to obtain similar information on a somewhat smaller scale by the isolation of phages from the whey and testing these for ability to lyse the starters in use. A centralization of all the data obtained might yield more valuable and complete information, especially if such an arrangement was carried out in close co-operation with a nearby cheese-making factory.

VI. SEROLOGICAL CLASSIFICATION OF PHAGES

It has already been suggested that many phage types of lactic streptococci exist. It seemed that interesting data might be obtained by a serological classification of the test phages employed for typing the strains. If any relationship existed between the serological reactions and phage-organism reactions of the phages, this could be regarded as valuable confirmatory evidence in favour of the suggested division of strains into phage types, whilst an absence of relationship would not detract from the value of results previously obtained.

Burnet (66) showed that sera prepared for phages, lysing dysentery-coli strains, could be titrated against other phages by decrease in plaque count. This method appears to be reasonably reproducible, and Rountree (67) has applied it successfully to staphylococcal phages.

Phages have also been classified serologically by Delbrück (68), but he and his colleagues worked almost exclusively with the 'T system' of phages for one strain of *E. coli*. They found that the classification fitted perfectly with certain morphological characters of the seven T phages. Delbrück's results have only a limited application, since the work was confined to phages lysing one strain only.

Evans (44) differentiated four distinct serological races of phages for haemolytic streptococci. The potency of the antiphage sera was tested in liquid medium. She showed that the haemolytic streptococci could be divided by their reactions with four serological races of phages 'into groups which corresponded approximately with groups that have been recognized on the basis of other characters'.

Whilst working with starter strains Whitehead & Hunter (63) prepared a serum for a phage

*rw*1, developed on a resistant strain after the original strain RW had been lysed by phage *rw*. A serum was also prepared for phage *rw* in order to find whether any common antigen existed in the phages *rw*1 and *rw* which might indicate a common origin. The sera prepared from phages *rw* and *rw*1 had a mutually inactivating effect indicating that the phages were antigenically related, but the same sera tested against phage *r*, of a distinct phage group, had practically no effect.

No previous attempts have been made to classify the phages for the lactic streptococci and it was decided to attempt the preparation of sera for phages lysing *Str. cremoris* and *Str. lactis* strains.

1. TECHNIQUE

(i) *Preparation of antiphage sera*

The method was essentially that used by Burnet (66) and by Rountree (67). Antiphage sera were obtained by injecting Seitz-filtered suspension of phage in Y.D.B. intravenously into rabbits. The phage suspensions were of the highest titre which could ordinarily be prepared for the particular phages on their homologous strains. At first ten phages were selected from the collection of test phages previously employed for typing the strains. Nine of the original phages selected were included in the indicative 'phage pattern' of one or other of the types. Sera were prepared for phages 144*f* and 167/11, which were both used for identifying the same type (VIII), and for phage 953/7, which, although reactive, was not type specific and was, therefore, not included in any 'phage pattern'. Later sera were prepared from a further four phages (p. 205). The response to the phage injections in rabbits was, with one exception, good.

A course of five injections was given at approximately 5-day intervals starting with 0·25 ml. quantities and increasing the quantity to 2·0 ml. The rabbits were bled (1–2 ml.) from the ear about 1 week after the last injection, and if the titre of the serum from this test bleeding was unsatisfactory further injections (2·0 ml.) of phage were given. When a satisfactory titre had been obtained the rabbit was bled (30–50 ml.), and the sera stored in the refrigerator without preservative.

(ii) *Titration of phages—selection of standard dilution*

It was necessary to standardize the titre of the homologous phage before sera could be tested.

At first, the standard selected was a dilution of the phage in Y.D.B. which, when mixed with equal quantities (0·2 ml.) of normal rabbit serum would, on testing, give a count of about 200 plaques per plate. The mixtures of phage dilutions and serum were kept at 30° C. until examined, 3¼ hr. later. The mixture was tested by spreading 0·02 ml. on a dry Y.D.A. plate previously smeared with a 24 hr. Y.D.B. culture of the homologous strain. Tenfold dilutions of phage were prepared by mixing 0·05 ml. of phage with 0·45 ml. of broth. All plate tests were duplicated throughout.

It was found later that, if the phage dilution chosen as the standard was adjusted so that the plaque counts per plate were greatly increased, the reading of results was facilitated. When the standard dilution gave a plaque count of about 200, a count had to be made on all the plates of a series in order to determine the effect of adding sera. If the standard dilution gave a high plaque count, the plaques had to be estimated only on those plates

showing partial neutralization of phage by the serum. Otherwise the results were obvious on inspection. The dilutions of phage selected gave abundant and, in many instances, uncountable plaques and also made some allowance for phages the titre of which fell on storage, since testing was spread over several weeks. Dilutions were selected for all, except four, of the seventy-eight test phages used for the phage typing. Some of these phages were used neat, since dilutions of phage either did not show an appreciable number of plaques or failed to reveal lysis. Certain phages always exhibited this characteristic, e.g. 314/13, 339/2, *s*338/2, *l*307/2, *ba*1, *b*22, *bw*2, *c*21, *c*23 and *c*31.

In the actual plating of the serum-phage mixtures the small volume of mixture (0·02 ml.) had to be spread rapidly, to prevent the mixture from drying in at the point of application. A light glass spreader, narrow glass tubing drawn out into a capillary and bent to form a spreader of about an inch in length, was found suitable for this purpose. Unscratched flat plates were, of course, necessary.

The pipettes were made and calibrated according to the suggestions of Wright & Colebrook (69). Four sizes to deliver the following volumes were made:

0·45 ml. 0·05 ml.	for diluting sera and phages.
0·2 ml.	for mixing sera and phages.
0·02 ml.	for dropping mixtures of sera and phages on to agar plates.

Glass spreaders, immediately after use, were sterilized by holding in boiling distilled water, dried in a bunsen flame and allowed to cool in a Petri dish. Pipettes were rinsed in boiling water, dried and allowed to cool inside a sterile 10 ml. tube.

(iii) *Titration of sera—selection of dilutions inactivated by homologous phages*

In order to determine the highest dilution of antiphage serum which would inactivate the standard dilution of homologous phage, equal volumes (0·2 ml.) of serum dilution or neat serum were mixed with the standard phage dilution already selected. (The serum was diluted using 0·05 ml. quantities in 0·45 ml. of broth.) The serum-phage mixtures were kept at 30° C., as before, and tested as above for the titration of the phage.

In all tests a control was set up by adding normal rabbit serum to the standard dilution of phage selected, and used to estimate the neutralizing effect of the antiphage sera.

(iv) *Titration of test phages for lactic streptococci against sera*

When the highest dilution of antiphage serum which inactivated the standard dilution of homologous phage was known, standard dilutions of the test phages were tested against the neat serum and against all the dilutions which inactivated the homologous phage. Eight sera were tested neat and in the 10^{-1} and 10^{-2} dilutions and six in neat and the 10^{-1} dilution only. Usually the neutralizing effect of several sera was tested at the same time against one phage, since the same controls served for several tests. In all some 5500 tests have been made. The results are summarized in Table 7.

(v) *Assessment of neutralizing effect of antiphage serum*

The technique for testing the neutralizing action of antiphage sera against a large collection of phages was complicated, and depended on two factors which in themselves were difficult to standardize: (*a*) the preliminary titration of the phage to be tested to obtain the

standard dilution; and (*b*) the titration of serum against the homologous phage to obtain the neutralizing dilutions.

Any variations in phage-organism relationships between each combination of phage and homologous strain would influence the results of (*a*) and/or (*b*). Yet some kind of quantitative approach was required to compare the effect of a particular antiphage serum on a collection of phages.

Burnet (66) considered that the serum had a positive neutralizing effect when the phage count was reduced to 5% of that of the control. Rountree (67) regarded a reduction of plaques to 10–20% as significant.

Assessing similar results with phages for the lactic streptococci, whilst all measurements in the titrations of the test phages against sera were carried out accurately, the final reading of neutralizing capacity of the antiphage sera has been on a qualitative rather than quantitative basis. This was necessary because the standard dilution of phage chosen often gave abundant plaques on the control plates (p. 201). Andrewes & Elford (70) found that 'over a very wide range a given dilution of serum neutralized in a given time an approximately constant percentage of phage, however much phage there was present'. A large excess of phage antibody seemed to be required for demonstrating any neutralization of phage and the controlling factor, therefore, was probably the extent of dilution of antiserum, rather than the degree of dilution of phage used. The results obtained with phages for the lactic streptococci confirmed the findings of Andrewes & Elford and, where the control plates had given uncountable plaques, the results were still easily observed when the serum reacted.

Three degrees of neutralizing power of the antiphage sera have been recorded (Table 7):

* inactivation of test phage (i.e. reduction of plaques to within 10% of control) by highest dilution of antiserum which inactivated homologous phage.

\+ partial reduction (10–80%) of plaque count of phage under test by highest dilution of antiphage serum inactivating homologous phage.

t appreciable reduction (50%) of plaque count of test phage by neat serum.

2. DISCUSSION OF RESULTS

(1) A general survey only could be made from the results of the serological typing of the test phages as a whole. Additional sera prepared from other phages are required before a final serological typing can be suggested for the phages of the lactic streptococci. The findings discussed below should be considered as a preliminary picture to be clarified or modified as further data are examined. The results have been considered in conjunction with the observations made on the phage typing of strains of lactic streptococci (pp. 183–91).

(2) When the phages were arranged according to the neutralizing power of the ten antiphage sera originally prepared they fell into three groups, the phages reacting with homologous sera for (i) phages 1*p*5, *b*/3, 144*f*, 167/11, 318*b*/27 and *l*277/25; (ii) phage *c*8; (iii) phages *x*71, 653/7 and 953/7.

Burnet (66) reported that the serological classification of coli-dysentery phages could be related to classification based on particle size and plaque morphology. Rountree (67) found that the serological types of phages could be related to the strains of staphylococci they attacked and to certain other characteristics of the phages, e.g. stability and plaque size. Delbrück (68) mentioned that a serological grouping of the 'T' system of phages fitted perfectly with morphological findings.

Particle sizes have not been estimated nor have plaque measurements been made for the lactic streptococcal phages but, as a working knowledge of the phage-organism relationships has accumulated, information on characteristic plaque sizes of these phages has become available. Applying these rather general criteria, the phages mentioned under (i) readily formed plaques which were small and distinct whilst the plaques of phages under (ii) *c*8, and (iii) *x*71, 653/7 and 953/7 were, by comparison, very large. Hence, classed on neutralizing power of the antiphage sera, phages grouped together gave plaques of similar size. As Burnet & Rountree found, the serological typing of the phages appeared to follow plaque size more closely than phage-organism relationships. Phages *x*71, *a*2 and *a*4, lysing strains of type IX*b*, were similar in plaque size to phages *a*3 and 653/7, reacting with strains of type VI*a*, yet all these phages were neutralized by the same sera. Serum for 953/7, a phage which was non-specific in phage-organism relationships, inactivated all the phages forming large plaques with the exception of two phages, *x*71 and *t*3.

(3) During the phage typing of strains of lactic streptococci the sixty-three test phages were grouped together to give typical 'phage patterns' for the types. Eleven such groups of phages were formed corresponding to the eleven phage types of the strains. A few test phages could not be included in any of the groups, e.g. *l*340/1 and *a*5; an equally small number were included in more than one 'phage pattern', e.g. *m*3/1, *t*2 and 769/5 (Table 5). The phage antiserum reactions appeared to be less specific and segregated the fifty-nine test phages which reacted with the antiphage sera only into three main groups. As a result, one antiphage serum often neutralized, to some degree, test phages regarded as indicative of quite distinct phage types. For example, serum for phage 318*b*/27 reacted strongly with type I*a* 'phage pattern' (phages 318*b*/27, 314/13 and *l*307/2) in addition, with some of the phages indicative of types II (phage 324/2) and VI*c* (phage *s*338/2), and to a lesser extent with phages indicative of types V and VIII.

Whilst a perfect correlation could not be expected between the two different reactions, i.e. phage-antiserum and phage-organism, in which the only common factor was phage, it was possible that some correlation might exist between the two reactions. There was a remarkably close serological relationship between all the phages which were indicative of types I*a* and VIII*a* respectively, but in some 'phage patterns' not all the constituent phages were related serologically. The 'phage patterns' for types VI*b* and *c* (see below) both contained phages of two different serological groups, namely (i) and (iii).

	Serological group										
	(iii)						Not grouped	(i)			
Phages	769/5	267/1	799/11	653/7	*a*3	*a*1	810/7	*s*338/2	339/2	821/2	*p*8/1
Phage type VI*b*	+	+	+	+	+	+	+	+	+	−	−
Phage type VI*c*	+	+	+	+	+	+	−	+	+	+	+

Further, in type IX*a*, phages *m*3/1, *x*17, *n*35, *t*2 and *x*62 were not neutralized by antiphage sera for phages *x*71, 653/7 and 953/7 (iii), yet they were indicative phages for the type together with seven other phages which were neutralized by these sera.

Not all the phages in one 'phage pattern' were related serologically, so that one strain might be lysed by phages of different serological type. Strain R652/1 from type VI*b* was lysed by phages 267/1, 769/5 and 653/7 which were not affected by the antiphage sera of (i). It was also lysed by phage 810/7 which was not inactivated by any of the sera available

and by phage *s*338/2, neutralized by the sera of (i). Strain X17 (type IX*a*) was lysed by phage *x*17 which was not neutralized by the sera available and by phage *a*2 which was placed serologically with the phages of (iii).

(4) The ten antiphage sera prepared first did not react with ten of the phages, viz. *t*3, 701/2, *c*31, 810/7, *m*3/1, *x*17, *n*35, *t*2, *x*62 and *bw*2. All these phages (except the first mentioned) formed tiny plaques and were difficult to obtain as high-titre lysates and showed characteristics associated with large phage particles. Phage *t*3 formed large plaques and it was unlikely to be grouped with the other nine phages not reacting with the sera prepared, nor was it related serologically to the other test phages giving similar plaques. Sera prepared for four of these ten phages, viz. *t*3, 810/7, *bw*2 and *x*62, were found to be highly specific for their respective homologous phages and no further serological relationship was established between the ten phages not inactivated by the sera originally prepared (Table 7).

VII. SUMMARY

1. The present position in the problem of 'slow' starter due to phage lysis has been reviewed.

2. The sources of some 450 strains of lactic streptococci, collected from a wide field, are given. The methods used in their selection for use as starter together with storage conditions, suitable to maintain their activity, have been discussed.

3. The sources from which phages for the lactic streptococci have been obtained and the methods of 'building up' a phage of high titre from dairy products, have been discussed. Attempts have been made to isolate phages from pig-faeces, after feeding starter strains to pigs, to adapt phages to strains previously unattacked and to establish interrelationship of the phages and strains of lactic streptococci with phages and strains of enterococci.

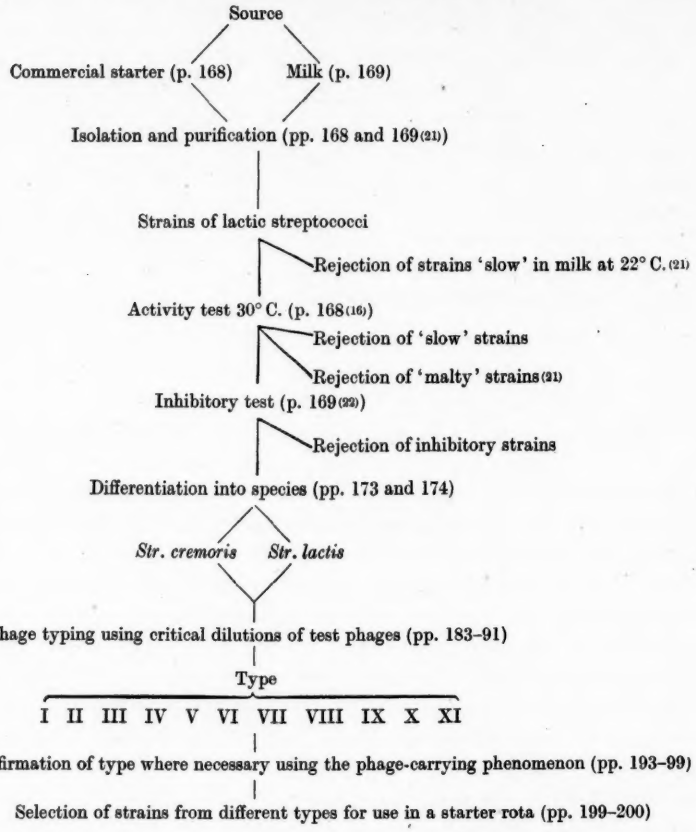
4. The strains of lactic streptococci have been classified in eleven phage types by modifying a method described for the phage typing of staphylococci. Identification of a type has been based on the reactions of a 'phage pattern' and not on the reaction of a single phage. It has not been possible to adapt one phage to lyse all the strains of one type and to replace the several phages on which type identification originally depended but, adapted phages have indicated the subdivision of some types.

5. The application of the 'phage-resistant carrier' strain phenomenon to phage typing has been investigated. The results have helped in the typing of some strains.

6. Phages used in typing the strains have been classified by means of antiphage sera, prepared from a selection of the test phages. On the data presented the majority of the phages were divided into three groups and the results have been discussed with reference to the phage types suggested.

7. The significance of phage typing, adaptation of phage and phage carrying in the selection of starter strains has been considered and the best method of applying this information to the control of 'slowness' in cheese factories has been discussed.

Important points in selection of starter strains


Source
Commercial starter (p. 168)
Milk (p. 169)
Isolation and purification (pp. 168 and 169(21))
Strains of lactic streptococci
Rejection of strains 'slow' in milk at 22° C.(21)
Activity test 30° C. (p. 168(16))
Rejection of 'slow' strains
Rejection of 'malty' strains(21)
Inhibitory test (p. 169(22))
Rejection of inhibitory strains
Differentiation into species (pp. 173 and 174)
Str. cremoris
Str. lactis
Phage typing using critical dilutions of test phages (pp. 183–91)
Type
I II III IV V VI VII VIII IX X XI
Confirmation of type where necessary using the phage-carrying phenomenon (pp. 193–99)
Selection of strains from different types for use in a starter rota (pp. 199–200)

Before strains, selected as above, can be included in a starter rota their suitability for cheese-making under commercial conditions must be tested in the vat.

Note. The 'nascent' phage phenomenon has not been included above since it has not been discussed in this paper.

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388. THE COLORIMETRIC DETERMINATION OF LACTIC ACID IN MILK AND MILK PRODUCTS

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(With 2 Figures)

There is a need for a rapid, yet accurate method for the direct determination of lactic acid in milk and milk products. The limitations of the simple estimation of titratable acidity are well known, and a direct method would be of great value in the assessment and control of the quality of milk and milk products, and in studies of the metabolism of the lactic acid bacteria.

For many years the method most commonly employed for the determination of lactic acid in biological materials was based on the principle of oxidation to acetaldehyde, distillation of the latter into bisulphite solution, and estimation of the bound bisulphite by iodine titration. This method, successfully applied to milk by Troy & Sharp (1) is, however, long and tedious, and Hillig later developed a method (2, 3) involving the extraction of lactic acid with ether from a protein-free filtrate, the removal of interfering substances, development of a colour with ferric chloride, and comparison with standards. This method has now been made official by the Association of Official Agricultural Chemists (4). Meanwhile, Heinemann (5) adapted for use with milk the method of Mendel & Goldscheider (6) for blood, in which lactic acid in a protein and glucose-free filtrate was oxidized by sulphuric acid to acetaldehyde, a colour formed between the acetaldehyde and veratrole, and a comparison of the colour made with standards. Heinemann reported an accuracy of ± 4 mg. % of the milk, but stressed the difficulties encountered in obtaining reproducible colour development.

In 1941 Barker & Summerson (7) adopted the *p*-hydroxydiphenyl reaction of Eegriwe (8) in preference to the veratrole reaction for the estimation of lactic acid in blood and tissues. Their procedure involved removal of glucose from a protein-free filtrate by the copper hydroxide-calcium hydroxide method of Van Slyke (9), oxidation of lactic acid to acetaldehyde in concentrated sulphuric acid, development of a purple colour between acetaldehyde and *p*-hydroxydiphenyl reagent, and comparison of the colour intensity with that developed from standard lactate solutions. This convenient and rapid method, which was unaffected by a large number of organic compounds related to lactic acid, appeared to provide the type of method needed, and the object of this paper is to describe its successful application to milk and milk products.

DESCRIPTION OF METHOD

Preliminary experiments

In preliminary experiments the procedure of Barker & Summerson was applied to milk exactly as described for blood, but later the removal of protein and lactose was simplified by adopting the procedure described by Troy & Sharp (1) in which protein and lactose were precipitated together by means of copper sulphate and calcium hydroxide. Other stages

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are essentially the same as those of Barker & Summerson, and the resulting method is described in full below.

It is realized that the greater part of the lactic acid formed by bacterial action is present in milk as lactate, but the usual practice is followed of expressing the results as lactic acid.

Reagents

All reagents should be of recognized analytical quality.

Copper sulphate solution (*a*), 25% (w/v) $CuSO_4.5H_2O$ in distilled water.

Copper sulphate solution (*b*), 5% (w/v) $CuSO_4.5H_2O$ in distilled water.

Calcium hydroxide suspension prepared by grinding 300 g. calcium oxide with water to a cream in a mortar, and transferring to a container with water until a total of 1400 ml. of water has been added.

Sulphuric acid conc., specific gravity 1·84.

p-Hydroxydiphenyl reagent, 1·5% (w/v) in 0·5% aqueous sodium hydroxide, prepared by dissolving 1·5 g. in 10 ml. 5% (w/v) sodium hydroxide, heating and stirring till dissolved, then making up to 100 ml. Store in a dark glass bottle.

Primary standard solution. Dissolve 0·1067 g. lithium lactate in 1 l. distilled water immediately before use.

Working standard solution. Add 3, 6, 9, 12 ml. respectively of the primary standard solution to four 100 ml. graduated flasks and make up to the mark with water. These solutions contain the equivalent of 3, 6, 9 and 12 μg. lactic acid per ml.

Preparation of protein and lactose-free filtrate

Dilute 5 ml. of milk or reconstituted milk product with water to approximately 35 ml. in a 50 ml. graduated flask. Add, while swirling, 0·5 ml. 5% copper sulphate solution. Raise the temperature of the flask contents to 45–47° C. by rapid swirling in a water-bath at approximately 90° C. and, after addition of 5 ml. 25% copper sulphate solution, hold for 10 min. at 45–47° C. Add 5 ml. calcium hydroxide suspension, make up to the mark with water and add a further 0·2 ml. to allow for contraction when cooled later to 20° C., shake vigorously, and hold for a further 10 min. at 45–47° C. Cool to 20° C. in running tap water and filter.

If this primary filtrate is left for more than 1 hr., there is a tendency for calcium sulphate to precipitate, but it does not interfere with subsequent procedure.

The filtrate from milk which has undergone appreciable proteolysis may show a purple colour due to the biuret reaction, but the colour disappears on subsequent addition of the sulphuric acid, and has not been found to interfere.

A filtrate containing between 1 and 10 μg. lactic acid per ml. is required, and filtrates containing greater amounts must be diluted accordingly. If the filtrate has an unknown concentration, a ten-times dilution of the primary filtrate is most suitable as a first trial. This may later be changed to 1, 20, 50 or 100 times, as necessary.

If it is known that a sample of milk will contain between say 10 and 100 mg./100 ml., then a filtrate containing the required 1–10 μg. lactic acid may be prepared directly by taking 1 ml. milk in a 100 ml. flask and carrying out the protein and lactose precipitation using 0·5 ml. 5% copper sulphate, 2 ml. 25% copper sulphate, and 5 ml. calcium hydroxide suspension.

Primary filtrates which will require considerable dilution have been kept for several hours at room temperature, or overnight in the refrigerator, without loss of lactic acid, but diluted filtrates containing less than 10 μg. per ml. tended to lose lactic acid and should be estimated as soon as possible.

Precipitation at room temperature has been carried out. The filtrate obtained was generally light purple in colour even with fresh milks, and although close agreement with the results found when employing precipitation at 45° C. was sometimes obtained, there was a tendency towards low figures. The method of precipitation at 45° C. was preferred and is recommended.

Development of colour

Measure 1 ml. filtrate containing 1–10 μg. lactic acid into a pyrex tube 1 in. × 6 in. Add one drop 5% copper sulphate, which as Barker & Summerson have shown, ensures maximum colour development and minimum interference from cations, and add from a burette exactly 6 ml. concentrated sulphuric acid. Place the tube in boiling water for 5 min. and cool to 20° C. in iced water. Add two drops *p*-hydroxydiphenyl reagent, agitate thoroughly to ensure a fine suspension of the reagent which is insoluble in acid, and hold at 30 ± 2° C. for 15 min. Add a further drop of reagent, mix well again and hold for a further 15 min. at 30° C. Transfer the tubes to a boiling water-bath for 90 sec. to dissolve excess suspended reagent, and cool to room temperature in iced water prior to reading in a suitable absorptiometer using a 1 cm. cell, or in a colorimeter.

Russell (10), investigating irregularities in the Barker & Summerson method, found that some batches of sulphuric acid were unsatisfactory. One source of error was found to be the presence of nitrate and nitrite ions in the acid, and workers were advised to pay particular attention to the nitrate and nitrite contents of any sulphuric acid used for this method. In our experience no difficulty has been found with various batches of sulphuric acid of analytical reagent quality.

Preparation of colour standards

One ml. each of the working standard solutions containing 3, 6, 9 and 12 μg. lactic acid are pipetted into 1 in. × 6 in. pyrex tubes and colours developed as described above for the milk filtrates.

Measurement of colour

Barker & Summerson made use of the spectrophotometric data of Block & Bolling (11) who show, in a graph plotting absorption factor against wave-lengths between 400 and 750 mμ, that the maximum absorption is at approximately 560 mμ. In order to ascertain this more closely an experiment was carried out using a photoelectric spectrophotometer, to find the absorption between 540 and 590 mμ, using wave-length intervals of 10 or 5 mμ. As can be seen from Fig. 1 the maximum was found to be at 570 mμ.

When the colour standards were measured in the spectrophotometer using a 1 cm. cell and wave-length 570 ± 5 mμ, a linear relationship was obtained between extinction and lactic acid concentration as shown in Fig. 2.

The majority of measurements, however, were carried out with the Miller absorptiometer based on the design of Morris (12), using Ilford spectrum filter number 605, the yellow-green, with maximum transmission at around 555 mμ. With this intrument the extinction values $E_{1\text{ cm.}}$, obtained for 3, 6, 9 and 12 μg. lactic acid were 0·226, 0·442, 0·652 and 0·838 respectively, giving a curvilinear relationship.

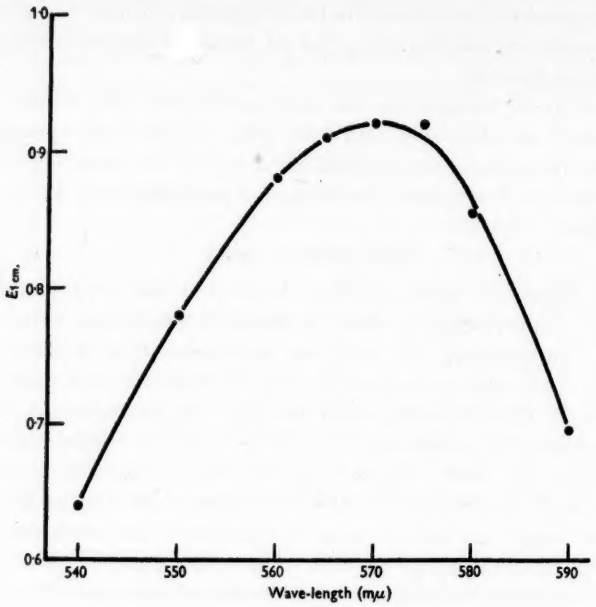
1·0
0·9
0·8
0·7
0·6
$E_{1\,cm.}$
540
550
560
570
580
590
Wave-length (mμ)

Fig. 1.

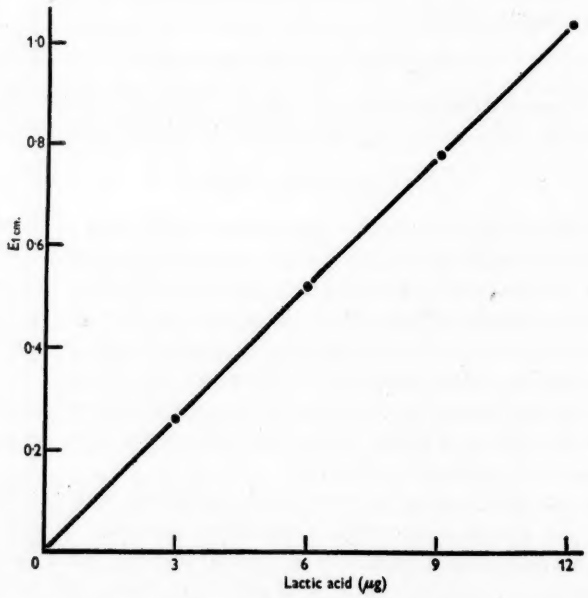
1·0
0·8
0·6
0·4
0·2
0
$E_{1\,cm.}$
3
6
9
12
Lactic acid (μg)

Fig. 2.

Calculation of results

A value is obtained for the extinction of the unknown solution and also one for the extinction of the appropriate reagent blank prepared by mixing the reagents, diluting to the same extent, and subjecting to the described procedure. The corresponding values for lactic acid are then read off the standard curve. There are two correction factors to be applied after due allowance has been made for milk dilution: (*a*) for the volume occupied by, and (*b*) for the partial retention of lactic acid by, the precipitate in the 50 ml. flask.

(*a*) In this 50 ml. graduated flask a volume of approximately 1 ml. is occupied by the copper-calcium hydroxide precipitate and an average of 0·3 ml. by the protein and fat. The nett volume of liquid is thus approximately 48·7 ml. or 97·5% of the total volume. A multiplication factor of 0·975 must therefore be applied. The volume occupied by protein and fat is only a small proportion of the total precipitate and so the effect of normal variations in the composition of milks will be negligible when the average factor is applied to particular milks. It is clear, however, that for products such as cream, where a high fat content is expected, the factor allowing for volume of precipitate will have to be adjusted.

(*b*) When the precipitation procedure involving copper sulphate and calcium hydroxide is used there is a small retention of lactic acid by the precipitate. As Troy & Sharp have shown, this retention is principally due to lactose in the precipitate and for all levels of lactic acid leads to recoveries of 90–94% with an average of 92%. As is shown later this is in agreement with our findings and a correction factor of 100/92 is necessary.

By combining the above two factors it follows that an overall multiplication factor of 1·06 should be applied to all results.

ACCURACY OF THE METHOD

Recovery experiments

A number of recovery experiments were carried out in which various levels of lactic acid from 20 to 400 mg./100 ml. were added to fresh raw milk. Ten, twenty or fifty times dilution of the primary filtrates was required. The recovery at all levels varied between 96

Table 1

Lactic acid added (mg./100 ml.)	Recovery (mg./100 ml.)	Recovery (%)
20	19·7	99
40	41·1	103
60	57·4	96
100	97·7	98
140	141·2	101
160	162·0	102
200	200·7	100
400	400·8	100

and 103% with an average of 100%. This variation is due partly to the actual method of determining the lactate in the prepared filtrates, and partly to the general application of the factor 100/92 to correct for the adsorption on the precipitate, when the factor for the particular sample might well have been between 100/90 and 100/94. The resultant accuracy is, however, satisfactory and adequate for the correct interpretation of results.

Experiments conducted at levels of addition below 10 mg./100 ml., where no dilution of the primary filtrate is required, gave recoveries which, even after applying the above

correction factors, were consistently low by 10–15%, but which were, nevertheless, within 0·5–1 mg./100 ml. Where greater accuracy is desired below the 10 mg. level a factor of 1·25 instead of 1·06 has been found suitable.

With regard to reproducibility of the technique, the average difference between duplicates in any given determination was 2%.

Comparison with the distillation method

A comparison with the established distillation procedure of Troy and Sharp was carried out. Samples of fresh raw milk were incubated at 30° C. with a strain of *Streptococcus cremoris*, and the acidity followed by titration until the desired acidity was reached. Filtrates were then prepared by the copper sulphate-calcium hydroxide procedure, and lactic acid estimated by the distillation and *p*-hydroxydiphenyl methods. Giving the Troy & Sharp figures first and *p*-hydroxydiphenyl figures second, the results in mg. developed lactic acid per 100 ml. of milk, were 24·6 and 26·3; 35·7 and 34·0; 42·5 and 41·4; 43·5 and 41·3; 48·3 and 49·1; 67·3 and 69·5. These figures show close agreement.

APPLICATION OF THE METHOD TO MILK AND MILK PRODUCTS

Apparent lactate content of fresh milk

Fresh raw milk contains an inherent small amount of lactic acid. Troy & Sharp (1) found an average of 2 mg.% by the distillation method and Gould (13) found between 1 and 3 mg.% by the Hillig ether extraction procedure. Many samples of fresh raw milk, tested by the *p*-hydroxydiphenyl method, were found to contain 1·5–2·0 mg./100 ml., which is in close agreement with the above figures.

It should be pointed out that, owing to the presence of this initial lactic acid, the value obtained when the method described is applied to milks containing developed lactic acid represents the total lactic acid present, and the developed will, of course, be some 1·5–2·0 mg./100 ml. lower.

Milk powder

One of the merits of the present method is its suitability for the routine determination of lactic acid in milk powders. Two fresh milk powders known to have been prepared for experimental purposes without delay from milk produced under very satisfactory conditions were found to contain 20 and 21 mg. lactic acid % respectively. On a basis of 12·5% milk solids this corresponds to a milk containing 2·5 mg.% which is in close agreement with fresh milk figures. The method was also successfully applied to commercial samples of whole and skim milk powders containing various amounts of lactic acid up to more than 1000 mg.%.

It has been reported that milk powders from neutralized and over-neutralized milks give low results by the distillation method (14) and slightly high results by the Hillig ether extraction method (13). To obtain information on this point regarding the present method, a sample of whole milk powder was prepared from milk of high titratable acidity which had been neutralized back to its original acidity with bicarbonate, and roller dried immediately after neutralization. The liquid milk before and after neutralization was found to contain 150 and 148 mg. lactic acid/100 ml. respectively. The milk powder was found to contain 1316 mg./100 g. of powder, equivalent to 149 mg./100 ml. of liquid milk on the basis of the total solids content of the original milk which was 11·1%, showing that

neutralization and drying do not affect the accuracy of results obtained by the method herein described.

The difficulty of detecting the presence of added neutralizer in milk powder is well known, and the rapid determination of lactic acid in conjunction with a measurement of the pH of the reconstituted milk, should prove a useful supplement to the present methods for the detection of neutralizer such as the recommended alkalinity of the ash (4).

Other products

Although it has been applied as yet only to milk and milk powders, this method should also be applicable to whey, whey powder, buttermilk, cream and unsweetened condensed milk; these products being reconstituted where necessary to a normal liquid basis before carrying out the estimation.

DISCUSSION

The accuracy, ease of manipulation and speed with which the present method can be carried out simultaneously on large numbers of samples, renders it a desirable method for use in both routine and research work. One estimation may be carried out in 1½–2 hr., and a group of ten or twelve may conveniently be tested at the same time.

By comparison, the distillation method of Troy & Sharp is long and requires considerable skill. A fading end-point makes the final titration slow and uncertain, only one or two estimations can conveniently be run at the same time and, as Hillig found, neutralized and over-neutralized powders give low results by this method (14).

As co-operative trials showed (15), the Hillig ether extraction procedure with its final colorimetric measurement is accurate and reliable. Once again, however, the method is long and a more rapid one would be of great advantage. As Gould (13) has pointed out: 'the time and skill required for the determination may limit its final application'.

The present method gives results in close agreement with those found by the distillation method, and the distillation method was found by Hillig (14) to give satisfactory agreement with his own extraction procedure when neutralizer is absent.

SUMMARY

A method is described for the colorimetric determination of lactic acid in milk and unsweetened milk products. The protein and lactose is removed after precipitation with copper sulphate and calcium hydroxide at 45° C., the lactic acid in an aliquot of the filtrate is oxidized to acetaldehyde in sulphuric acid in the presence of copper sulphate, a purple colour is developed with *p*-hydroxydiphenyl, and the colour intensity is measured in a photoelectric absorptiometer or other suitable instrument.

Recoveries of from 96 to 103%, averaging 100%, were obtained when 20–400 mg. lactic acid/100 ml. were added to fresh milk.

The proposed method has an advantage over existing methods in the ease and speed with which large numbers of determinations can be carried out simultaneously whilst retaining the essential accuracy.

The author wishes to thank Dr S. J. Rowland for valuable advice and interest during this work.

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637.127 (62)

389. THE COMPOSITION OF THE MILK OF EGYPTIAN COWS AND BUFFALOES*

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(With 5 Figures)

Although the composition of cows' milk has been thoroughly studied in many countries, it is still open to discussion and much work has to be carried out under Egyptian conditions until a definite conclusion regarding its properties can be arrived at.

As regards buffaloes' milk there exist some scattered data dealing with its composition. In 1914 Pappel & Hogan (1) published results of their study on buffaloes' milk in the Egyptian Department of Public Health in Cairo. Also some other workers as Bunce (2), Fleischman, cited by Elsden & Walker (3), and Pappel & Richmond, cited by Elsden & Walker (3), contributed, some time ago, to the subject in other countries. Recently, however, Raffaelli (4), Buruiana & Efimov (5), Mihaeloff (6), Ghosh & Datta Roy (7), Maymone (8), and Anantakrishnan, Dastur & Kothavalla (9) have all investigated various aspects of the composition of buffaloes' milk. Unfortunately, these studies are still of little avail to dairy industry and legislation in Egypt. Therefore many attempts are still needed in order to establish an adequate knowledge of the various properties of the milk of these popular animals. The present study is thus one of these attempts contributing to the subject under conditions prevailing in Egypt.

EXPERIMENTAL

This study was carried out on bulk samples taken from both cow and buffalo herds of the Faculty of Agriculture at Giza, Egypt, throughout the year. Also individual milk samples were taken from 100 cows and the same number of buffaloes from Cairo, Giza and their neighbourhood; they were all taken at random. Individual sampling was evenly distributed throughout the year, while the interval between each two successive bulk samples was 2 weeks. All samples, either bulk or individual, were composite samples of both milkings during the day.

The two herds of the faculty were under good management. All animals were free from clinical cases of disease, and the average number of milking animals was 34 in the cow herd and 28 in the buffalo herd. Milking was by hand at 7 a.m. and 3 p.m.

During winter the two herds were fed on *Trifolium alexandrinum* (berseem) during the daytime and small amounts of wheat straw in the evening. Animals which yield more than 25 lb. received additional concentrated rations of barley, beans, cotton-seed cake, flaked maize and rice polish. This started on 5 December and continued until the 14 June.

Dry summer feeding began on 15 June. Animals received corn-fodder during the daytime, while at night they had straw in addition to the basic concentrated ration.

From these samples the following data were obtained: specific gravity at 60° F., fat, solids-not-fat, total nitrogen, and casein nitrogen. The longest period elapsing between

* Part of a thesis for degree of M.Sc in Fouad 1st University submitted by H. A. Hassan and supervised by A. M. El-Sokkary.

milking and the start of chemical analysis was 2 hr. for the portion of the sample taken from the morning milking and 17 hr. for the evening portion of the sample; this, however, was kept during this period at 4° C.

METHODS OF ANALYSIS

The specific gravity was estimated by an accurate Quevenne lactometer; correction was made by Richmond's scale for temperature.

Fat was determined by the original Gerber method, solids-not-fat by the usual method of evaporating 2 g. of milk, and total and casein nitrogen by the semi-micro method described by Rowland (10).

RESULTS

A summary of the analytical data is given in Table 1, while Tables 3 and 4 show the difference in composition between cows' and buffaloes' milk, for the individuals and for herd samples respectively. Ranges of variation, means, standard deviations, and standard errors of means were calculated. The significance of differences in the composition of both milks was tested by the *t*-test, which was carried out by means of Fisher's tables (11, 12). Each difference, the probability, or *P*, of which was 0·05 less, was considered significant.

Table 1. *Composition of individual and herd samples of buffaloes' and cows' milks*

(Composite morning and evening samples)

	Value	Individual sample (100 cows)	Cow herd	Individual sample (100 buffaloes)	Buffalo herd
Sp.gr. at 60° F.	Maximum	1·0398	1·0357	1·0368	1·0379
	Minimum	1·0284	1·0307	1·0285	1·0307
	Mean	1·0331	1·0337	1·0325	1·0339
	S.D.	0·0024	0·0014	0·0018	0·0020
Fat (%)	Maximum	7·60	5·15	10·20	7·50
	Minimum	2·05	3·60	4·25	5·50
	Mean	4·57	4·29	6·64	6·60
	S.D.	1·22	0·32	1·14	0·48
Solids-not-fat (%)	Maximum	10·19	9·52	11·30	10·58
	Minimum	8·18	8·66	8·90	9·43
	Mean	9·25	9·03	9·92	9·89
	S.D.	0·45	0·18	0·46	0·27
Total N (%)	Maximum	0·834	0·558	0·793	0·690
	Minimum	0·412	0·446	0·500	0·555
	Mean	0·557	0·509	0·616	0·621
	S.D.	0·078	0·028	0·066	0·035
Casein N(%)	Maximum	0·641	0·455	0·656	0·558
	Minimum	0·327	0·355	0·412	0·448
	Mean	0·442	0·405	0·501	0·504
	S.D.	0·063	0·025	0·055	0·025
Casein no.	Maximum	85·50	81·60	86·90	84·80
	Minimum	72·00	77·40	75·40	78·80
	Mean	79·34	79·60	81·34	81·20
	S.D.	2·20	1·00	1·83	1·64

The seasonal variations, in the composition of milk studied in both herds, are shown graphically in Figs. 1–5.

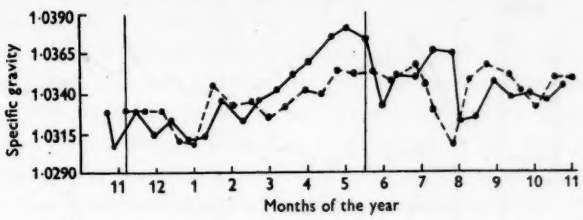

Fig. 1. Seasonal variations of the specific gravity. Continuous line, buffaloes; broken line, cows

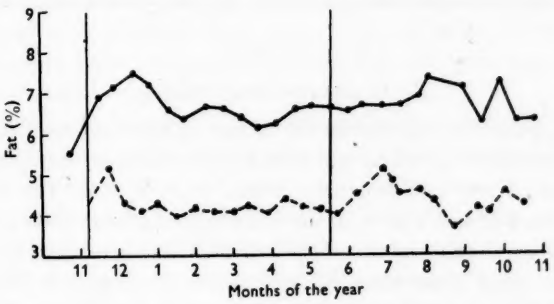

Fig. 2. Seasonal variations of fat content. Continuous line, buffaloes; broken line, cows.

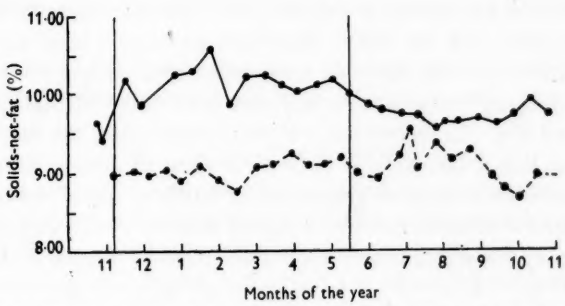

Fig. 3. Seasonal variations of solids-not-fat content. Continuous line, buffaloes; broken line, cows.

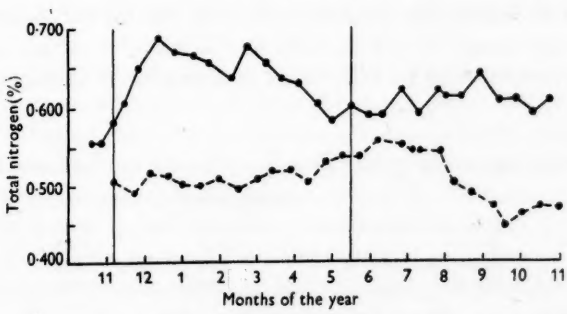

Fig. 4. Seasonal variations of total nitrogen content. Continuous line, buffaloes; broken line, cows.

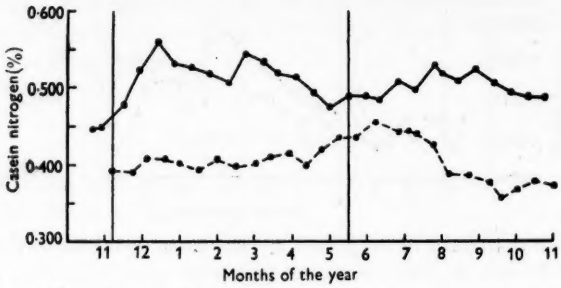

Fig. 5. Seasonal variations of casein nitrogen content. Continuous line, buffaloes; broken line, cows.

DISCUSSION

A. *Average composition*

From Table 1 it appears that the average fat content of Egyptian cows' milk is higher than all the averages, previously given by many workers, for different European breeds, except the Jersey, Welsh, Kerry and Guernsey breeds (3a, 13, 14, 15, 16). The average with the Egyptian 'breed' is 4·57 and 4·29% in the individual and herd milks respectively. With buffaloes' milk the mean values obtained for individual and herd milks are 6·64 and 6·60% respectively, thus being lower than those obtained by Pappel & Hogan (1), Bunce (2), Fleischman (3) and Maymone (8), but on the other hand they are higher than those given by Pappel & Richmond (3).

The mean percentages of solids-not-fat are, as seen in Table 1, 9·25 for individual cows, 9·03 for cow herd, 9·92 for individual buffaloes, and 9·89 for buffalo herd. It is clear that figures given for cows' milk are higher than those given by a large number of previous British investigators, but they agree to some extent with those given by Americans (16). Comparing the solids-not-fat value in the Egyptian cows' milk with that given by European breeds, it is found that the former has a higher content than the Shorthorn, Ayrshire, Friesian and Red Poll milks, while it approaches those of Jersey, Kerry and Guernsey breeds. As regards the average concentration in buffaloes' milk, one observes that the present results agree with those given by Pappel & Hogan (1), while they are slightly higher than those given by Fleischman, and lower than those of Pappel & Richmond (3), and Bunce (2).

Both mean values of total and casein nitrogen content obtained for cows' milk are slightly greater than those reported by Davies (15, 17), Golding, Mackintosh & Mattick (18, 19), Rowland (20) and El-Sokkary (21). Applying the factor 6·38 for calculating the percentage of total protein and casein, though its value for the former is not accurate, from values obtained in the present study for both values of nitrogen, the averages given in Table 2 are obtained.

Table 2. *Average percentages of total Protein and Casein in Cows' and Buffaloes' milks*

	Total protein (%)	Casein (%)
Individual cows	3·55	2·82
Herd	3·25	2·58
Individual buffaloes	3·93	3·20
Herd	3·96	3·22

By comparing the above values given for cows' milk with other results obtained by

previous investigators it is seen that while they are higher than some results they are lower than others (16a).

With buffaloes' milk, however, it is found that Pappel & Hogan (1), Fleischman (3), Pizzi (3) Buruiana & Efimov (5), and Mihaeloff (6) gave higher results than those shown above, while results given by Pappel & Richmond, and Stromer & Leather agree well (3).

Considering the casein nitrogen content as a percentage of the total nitrogen (casein number), the averages for cows, herd and individuals, are 79·60 and 79·34 respectively. Davies gave for this figure an average of 76·10 in one series and 76·7 in another (15, 17). Golding *et al.* reported the value of 76·5 (18, 19). Tocher 76·10 (22) and Rowland 78·70 in the case of individuals and 78·30 in the bulk samples (20). From these findings it is deduced that the average casein number of Egyptian cows' milk is higher than any of those given by previous authorities for European cows' milk. This may be due to the fact that either disease is less frequent among Egyptian cows or that the secreting tissues are more efficient with these animals than with the European breeds.

The casein number for buffaloes' milk; i.e. 81·34 for individuals and 81·20 for herd milk, is higher than that for cows', a fact which may be explained on the same lines as above with regard to the difference between Egyptian and foreign breeds.

As regards the specific gravity, it is observed from Table 1 that the values obtained are higher than those usually given for foreign breeds, the latter being 1·028–1·034 (average 1·032) for herd milk and 1·0135–1·0397 for individual samples (3); also the average value given by Tocher (22) is 1·0314, ranging from 1·026 to 1·036. This difference between the Egyptian and foreign milks may be explained by the higher solids-not-fat content of the former, as although fat, which lowers the specific gravity, is higher in the former milk, the increase in solids-not-fat content of this milk seems to be more than enough to counteract the effect.

The average results for the specific gravity of buffaloes' milk, particularly that of herd milk, are in harmony with the figure given by Zein-El-Abdeen (23), namely 1·0333.

B. *The frequency distribution*

Using the data obtained from the analysis of individual samples for working out the percentage distribution for each constituent, it is found, in general, that nearly half of the whole distribution is above the mode with both cows and buffaloes.

The highest percentage distribution of fat (41%) is within the range of 6·00–7·00% in the case of buffaloes' milk, while in cows' milk it is in the range of 4·00–5·00% (35%). For solids-not-fat 39% of the samples lie in the range of 9·40–9·80% with buffaloes', and 36% in the range of 8·80–9·20 with cows'; these represent the highest distribution in both cases.

In respect of the total nitrogen, 34% of the samples lie in the class 0·560–0·620% for buffaloes, while for cows, 30% of them lie in the class 0·490–0·550%; both being the highest classes. The respective classes in the case of casein nitrogen are 0·460–0·520 and 0·410–0·470%; representing 44 and 36% of all samples. As regards the casein number, the class 81·00–83·00 includes the highest percentage of the distribution, 47 %, for buffaloes, while for cows 41% lie in the class 78·00–80·00.

In the case of the specific gravity of buffaloes' milk the majority of the samples lie equally in the two classes 1·0310–1·033 and 1·0330–1·0350; this accounts for 66% of the population. With cows the highest percentage distribution, 36%, lies in the class 1·0320–

1·0340. A wider range of variation is reported for cows' milk than for buffaloes', but values for both milks overlap to a pronounced extent, particularly within the range of 1·028–1·035.

The present results are of considerable importance to Egyptian legislation concerned with milk. The present regulations require that the fat content of milk shall not be less than 3 and 5% in cows' and buffaloes' milk respectively. The present data show that 8% of the cows' samples and 3% of the buffaloes are below these legal minima. Also, according to these regulations, the solids-not-fat content of both milks should not be less than 8·5%; no samples, in the case of buffaloes' milk and 5% in the case of cows', fall below this standard. These findings show that the legal standards are reasonably founded, but that the legal minimum for solids-not-fat in buffaloes' milk should be raised to 9%.

C. *Difference in composition*

On inspection of Table 1 it is seen that the composition of individual milk samples differs from that of bulk samples in various respects. This is observed in both cows' and buffaloes' milk, but it is clear that these differences are more pronounced with cows than with buffaloes. In the former case the averages of specific gravity and casein numbers are respectively 0·0006 and 0·26% higher in the herd than in the individual samples, but, those for fat, solids-not-fat, total nitrogen, and casein nitrogen are 0·28, 0·22, 0·048, and 0·037% higher in the individual milk. In the case of buffaloes the averages in the bulk milk for total nitrogen and casein nitrogen are respectively 0·005 and 0·003% higher than in the individual milk, while those for fat, solids-not-fat, and casein number are higher with the individual samples than with bulk by 0·04, 0·03 and 0·14% respectively.

As regards the difference between the composition of cows' milk and that of buffaloes, Table 3 shows that, with the individual samples, the mean values of fat, solids-not-fat, total nitrogen, casein nitrogen, and casein number are higher for buffaloes than for cows by 2·07, 0·67, 0·059, 0·059 and 2·00 respectively, while the specific gravity is 0·0006 lower with the former than with the latter. All these differences are statistically significant. With the milk of the herds, the differences are also statistically significant excepting the value for specific gravity. Table 4 shows that buffaloes' herd-milk has on the average a much higher fat content, and solids-not-fat, total nitrogen and casein nitrogen are also higher than cows' herd-milk. Also the former has a higher casein number and specific gravity than the latter. The observed differences are 2·31% for fat, 0·86% for solids-not-fat, 0·112% for total nitrogen, 0·099% for casein nitrogen, 1·60 for casein number, and 0·0002 for the specific gravity.

Table 3. *Difference composition between cows' and buffaloes' milks*

Individual composite samples of morning and evening milks (100 cows and 100 buffaloes)

	Sp.gr. at 60° F.		Fat (%)		Solids-not-fat (%)		Total N (%)		Casein N (%)		Casein no.	
	C.	B.	C.	B.	C.	B.	C.	B.	C.	B.	C.	B.
Mean	1·0331	1·0325	4·57	6·64	9·25	9·92	0·557	0·616	0·442	0·501	79·34	81·34
S.D.	0·0024	0·0018	1·22	1·14	0·45	0·46	0·078	0·066	0·063	0·055	2·20	1·83
S.E.M.	0·0002	0·0002	0·12	0·11	0·05	0·05	0·008	0·007	0·006	0·006	0·22	0·18
Difference between the two means	−0·0006		+2·07		+0·67		+0·059		+0·059		+2·00	
t	1·9900		12·4073		10·3839		5·8009		7·0900		6·9775	
P	0·02–0·05		<0·01		<0·01		<0·01		<0·01		<0·01	
Sig.*	+		+		+		+		+		+	

* + = Significant difference. C. = cows. B. = buffaloes.

Table 4. *Difference in composition between cows' and buffaloes' herd-milk*

Bulk composite samples of morning and evening milks

	Sp.gr. at 60° F.		Fat (%)		Solids-not-fat (%)		Total N (%)		Casein N (%)		Casein no.	
	C.	B.	C.	B.	C.	B.	C.	B.	C.	B.	C.	B.
Mean	1·0337	1·0339	4·29	6·60	9·03	9·89	0·509	0·621	0·405	0·504	79·60	81·20
S.D.	0·0014	0·0020	0·32	0·48	0·18	0·27	0·028	0·035	0·025	0·025	1·00	1·64
S.E.M.	0·0003	0·0004	0·06	0·09	0·03	0·05	0·005	0·007	0·005	0·005	0·19	0·31
Difference between the two means	+0·0002		+2·31		+0·86		+0·112		+0·099		+1·60	
t	0·4284		20·7132		13·6538		13·0297		14·6011		4·3488	
P	0·6–0·7		0·01		0·01		0·01		0·01		0·01	
Sig.*	–		+		+		+		+		+	

* + = significant difference. C. = cows. B. = buffaloes.

From the above discussion one concludes that buffaloes' milk differs significantly from that of cows. This is of great importance from the point of view of differentiating between the two milks, which are most popular in Egypt. It also supplies us with valuable information when considering the suitability of each milk for the various branches of dairy manufacture.

Both milks are commonly sold for liquid consumption, and very often producers try to sell buffaloes' milk as cows' with the object of making profit. This is usually done after extracting some of the cream, which is sold at profitable rates. The appearance of the milk is altered to resemble that of cows' milk. Egyptian regulations differentiate between the two milks by their fat contents, the legal minimum standards being 3% for cows' and 5% for buffaloes'. This enables the producers to separate some cream from the latter and sell it as genuine cows' milk. This action is legally considered a method of adulteration, and thus presents considerable difficulty to legal and public health authorities.

The present study shows that the specific gravity test cannot be applied for differentiating between the two genuine milks. Although there is a significant difference between the two values in the case of individual samples, yet this difference becomes insignificant with the two bulked milks. Moreover, values overlap to a considerable extent.

Determinations of fat, solids-not-fat, total nitrogen, and casein nitrogen, together with the colour test (24) may, however, give reasonably reliable information for the differentiation required. Partially skimmed buffaloes' milk is easy to detect, because while it gives a fat figure corresponding to that of cows' milk, figures for solids-not-fat, total nitrogen and casein nitrogen will remain too high for genuine cows' milk.

D. *Seasonal variations*

Conditions in Egypt are, in many ways, different from those in Europe and America where many investigations have been carried out in connexion with the influence of seasonal variations on the composition of milk. Green feeding is usual in winter and dry feeding in summer, whereas in many other countries, where those investigations were made, the opposite is the usual practice. Also the Egyptian climate is rather warmer than in those countries. A study of those variations in this country is, therefore, found to be pertinent.

Figs. 1–5 show these variations for the specific gravity, fat, solids-not-fat, total nitrogen, and casein nitrogen contents. The two vertical lines in each graph indicate the dates of

feeding changes, the first, from the left, being for the commencement of feeding *Trifolium* (berseem) and the second for the change to dry summer feeding.

With regard to the specific gravity it is observed from Fig. 1 that the value in both cows' and buffaloes' bulked milks shows some irregular variations between the two levels, namely 1·0307 and 1·0327 from November to February, after which both curves tend to rise to the maximum of 1·0379 at the beginning of June with buffaloes, and 1·0357 on 25 July with cows. The curves then return to their irregular fluctuations, noticed at the beginning. These fluctuations at both periods are attributed, mainly, to new calvers joining the herd, and also to animals nearing the end of their lactation.

As for the concentration of fat, a tendency for both curves to rise at the beginning of the season is shown in Fig. 2; i.e. with buffaloes the curve rises from 5·50% on 22 November to 7·5% on 12 January, while with cows it rises from 4·30% on 7 December to 5·15% on 22 December. This may be due either to a large number of individuals in the two herds at the beginning of their lactation period, or to the quality of food given, as it follows the change from summer feeding to green winter feeding. After this, however, the curves drop to 4·30% on 4 January with cows and 6·55% on 8 February with buffaloes. Thereafter they remain more or less steady until 25 June with cows and 9 August with buffaloes. Subsequent to these dates the curves tend to rise for a short period, after which the buffaloes' curve shows a tendency to fall, with sharp fluctuations, while that of the cows returns to its previous level.

The fat content, therefore, seems to show no distinct variation with feeding changes. It is clear from the nature of both curves and from the dates on which fresh calvers entered both herds, that the stages of lactation mask the influence of food and seasonal changes. This is supported by the findings of many workers; Davies & Provan (25), Houston & Hale (26), Ragsdale & Brody (27), Hayes (28), and El-Sokkary (21*b*) as they all agree that food and season of the year have very little influence on the fat content of milk.

The inspection of Figs. 3–5 leads to an important observation, in that the variation in the composition of buffaloes' milk runs in the opposite direction to that of cows' milk. The highest concentrations during the year, for solids-not-fat, total nitrogen and casein nitrogen, are reached in summer with cows, while with buffaloes they are attained in winter. In the former case these maxima are 9·52% (3 August), 0·558 and 0·455% (5 July), respectively, while in the latter the respective values are 10·58% (22 February), 0·690 and 0·558% (11 January). It is also observed that with cows the curves maintain a higher level in summer than in winter, while with buffaloes the opposite occurs. In each case the three curves run closely parallel to each other.

As this influence is of a long duration with both buffaloes and cows, it cannot be attributed to feeding changes, since these are known to show a temporary effect of very short duration (16*c*, 21*c*, 29). It may therefore be attributed to the fact that buffaloes can endure the cold and changeable conditions of winter, and yield milk of a higher quality than under summer conditions, by which they seem to be more affected than the native 'breeds' of cows.

SUMMARY

The milk of 100 cows and of the same number of buffaloes, in Cairo and its neighbourhood, was analysed; analyses were also made on bulk samples taken at 2-week intervals from the milk of the two buffalo and cow herds of the Faculty of Agriculture at Giza. All samples were mixtures of morning and evening milkings in proportion to yield.

The following are the mean values and their standard errors for individual samples of both cows and buffaloes respectively:

	Cows		Buffaloes	
		S.E.M.		S.E.M.
Sp.gr.	1·0331	0·0002	1·0325	0·0002
Fat (%)	4·57	0·12	6·64	0·11
Solids-not-fat (%)	9·25	0·05	9·92	0·05
Total N (%)	0·557	0·008	0·616	0·007
Casein N (%)	0·442	0·006	0·501	0·006
Casein no.	79·34	0·22	81·34	0·18

For bulk samples the mean values are respectively as follows:

	Cows		Buffaloes	
		S.E.M.		S.E.M.
Sp.gr.	1·0337	0·0003	1·0339	0·0004
Fat (%)	4·29	0·06	6·60	0·09
Solids-not-fat (%)	9·03	0·03	9·89	0·05
Total N (%)	0·509	0·005	0·621	0·007
Casein N (%)	0·405	0·005	0·504	0·005
Casein no.	79·60	0·19	81·20	0·31

Almost all the differences between the composition of cows' milk and that of buffaloes are statistically significant. This is important from the point of view of differentiating between the two milks. Also by working out the frequency distribution for each constituent in both milks, different distributions and ranges of variation are observed.

Seasonal variations in composition are shown graphically. The most important observation on these variations is that with buffaloes' milk, the variation runs in an opposite direction to that of cows' milk. It is also established that, with each milk, the curves of solids-not-fat, total nitrogen, and casein nitrogen are always closely parallel to each other.

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390. THE ESTIMATION OF LOSSES OF BUTTERFAT IN CHURNING

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The extent of butterfat loss in churning is best expressed as a percentage of the amount of butterfat in the cream churned. Since weighing of the buttermilk run off is not practicable under commercial conditions of buttermaking it is necessary to use an indirect method of determining the quantity of buttermilk. This quantity comprises the amount of serum in the cream, less the amount of serum retained in the butter, and plus the amount of fat in the buttermilk. Udy (1, 2) concluded from analyses of butter (data not given) that butter contained about 10% of buttermilk, and from this premise he deduced the following formula for calculation of percentage fat loss in churning, given the percentage fat contents of the cream received (F) and of the unwatered buttermilk (f), and assuming a 20% overrun of finished butter manufactured:

$$\text{Percentage fat loss} = \frac{100 - 1{\cdot}12F}{F} \times \frac{f}{1} \quad \text{(Udy's formula).}$$

The formula was derived as follows:

$$\text{Butter from 100 parts of cream} = \frac{120}{100} \times F$$
$$= 1{\cdot}2F.$$

$$\text{Buttermilk in } 1{\cdot}2F \text{ parts of butter} = \frac{10}{100} \times 1{\cdot}2F$$
$$= 0{\cdot}12F.$$

Buttermilk run off from butter from 100 parts of cream = serum in cream − buttermilk in butter
$$= (100 - F) - 0{\cdot}12F$$
$$= 100 - 1{\cdot}12F.$$

$$\text{Quantity of fat in buttermilk from 100 parts of cream} = \frac{100 - 1{\cdot}12F}{1} \times \frac{f}{100}.$$

$$\text{Percentage fat loss} = \frac{\text{Wt. fat in buttermilk}}{\text{Wt. fat in cream}} \times 100$$
$$= \frac{100 - 1{\cdot}12F}{F} \times \frac{f}{100} \times 100$$
$$= \frac{100 - 1{\cdot}12F}{F} \times f.$$

Udy pointed out that the factor $\frac{100 - 1{\cdot}12F}{F}$ is constant for creams of any one percentage fat content, and he listed the values of this factor for creams of fat contents within the normal range at churning in commercial butter factories.

MODIFICATION OF UDY'S FORMULA

While the formula derived by Udy is sufficiently accurate for all practical purposes, it is in actual fact slightly incorrect, since in the calculation of quantity of buttermilk lost it does not allow for the presence of some fat in the buttermilk.

As stated above:

Quantity buttermilk lost = Quantity serum in cream − Quantity buttermilk in butter + Quantity butterfat in buttermilk.

Udy omitted the last term in this equation. It may be included in the formula as follows:

Quantity of buttermilk in butter from 100 parts cream $= 0{\cdot}12F$ (see above).

Quantity of fat in buttermilk in butter from 100 parts cream $= 0{\cdot}12F \times \frac{f}{100}$.

Hence quantity of serum in buttermilk in butter from 100 parts cream $= 0{\cdot}12F - \frac{0{\cdot}12F \times f}{100}$.

Now quantity of serum lost from 100 parts cream = Quantity serum in cream − Quantity serum in butter

$$= (100 - F) - \left(0{\cdot}12F - \frac{f \times 0{\cdot}12F}{100}\right)$$

$$= 100 - 1{\cdot}12F + 0{\cdot}0012fF,$$

and quantity of buttermilk $= \frac{100}{100 - f} \times$ quantity of serum in buttermilk.

Hence quantity of buttermilk lost from 100 parts cream $= \frac{100}{100 - f} \times (100 - 1{\cdot}12F + 0{\cdot}0012fF)$.

Therefore quantity fat in buttermilk lost from 100 parts cream $= \frac{f}{100} \times \frac{100}{100 - f} \times (100 - 1{\cdot}12F + 0{\cdot}0012fF)$,

and percentage fat loss $= \frac{\text{quantity fat in buttermilk}}{\text{quantity fat in cream}} \times 100$

$$= \frac{f}{100 - f} \times \frac{(100 - 1{\cdot}12F + 0{\cdot}0012fF)}{F} \times 100.$$

The effect of the modification on the result for percentage fat loss may be gauged from the following example:

Fat content of cream (%)	40
Fat content of buttermilk (%)	1·0
Percentage fat loss by Udy's formula	1·385
Percentage fat loss by modified Udy's formula	1·400

The difference is thus very small, and, in view of the possible errors in the estimation of fat content of the buttermilk, and also of the variations in proportion of buttermilk retained

in the butter (see below), the corrections involved in the modified formula are of little practical importance.

The term $0{\cdot}0012fF$ has no significant impact on the result for percentage fat loss, and any correction required to the result obtained by use of the Udy formula can be applied with sufficient accuracy by multiplying this result by the factor $\frac{100}{100-f}$.

Udy utilized his formula for estimation of fat loss over a day's churnings or over a composite-sample testing period, for which the average percentage fat content of the cream as received is known from the factory records. Cream is generally somewhat diluted with water during the processing in the factory. Udy estimated the extent of dilution by comparing the solids-not-fat content of the buttermilk with the average total solids content of the serum of cream received at the butter factory, which he found to be 8·8%. The solids-not-fat content of the buttermilk was calculated by Richmond's rule from the density reading in lactometer degrees and the fat content as determined by the butyl alcohol method. Then

$$\text{Percentage dilution} = \frac{\%\ \text{S.-N.-F. in buttermilk}}{8{\cdot}8} \times 100,$$

and

$$\%\ \text{fat in unwatered buttermilk} = \frac{\%\ \text{S.-N.-F. in buttermilk}}{8{\cdot}8} \times f.$$

Udy thus made an automatic allowance for all dilutions in processing, including the addition of break water during churning. He applied the method mainly to the study of the percentage fat loss as a factory average over the 10-day testing period, and for this purpose it is very satisfactory. It assumes, however, a constant rate of dilution of the cream with water on the farms, and is thus probably in error during the early spring and late autumn in New Zealand, when the quantity of water added is a greater proportion of the total cream supply from the farms, and as a result the solids-not-fat content of the serum of the cream as received is probably lower than 8·8%. Use of Udy's system for individual churnings is open to error because of possible variations in the solids-not-fat content of the unwatered buttermilk from the presumptive value of 8·8, and of possible errors in the application of the Richmond rule to different buttermilks. McDowall (3) has studied the magnitude of the effect of these possible errors on the result for percentage fat loss.

Bird & Derby (4) calculated the percentage fat loss from an individual churning of cream by the formula

$$\text{percentage fat loss} = \frac{100 - 1{\cdot}2F}{F} \times f,$$

where F represents the percentage fat content of the cream as churned, and f represents the percentage fat content of the buttermilk as run off.

Derivation of the Bird & Derby formula followed the principles described above for derivation of Udy's formula and, as in Udy's formula, the effect of fat content of buttermilk on the quantity of buttermilk is ignored. Their formula can be corrected for this omission by substituting 1·2 and 0·002 for the 1·12 and 0·0012 in the corrected Udy formula given above, i.e.

$$\%\ \text{fat loss in buttermilk} = \frac{f}{100-f} \times \frac{100 - 1{\cdot}2F + 0{\cdot}002fF}{F} - (\text{corrected Bird \& Derby formula}).$$

Wiley (5, 6) has pointed out that there is a discrepancy between the Udy and the Bird & Derby formulae, since, if the cream at churning is not diluted with water, *f* in Bird & Derby's formula will represent the fat content of the unwatered buttermilk, and the two formulae should be identical, whereas Udy's formula uses the factor 1·12 and the Bird & Derby formula the factor 1·2. The difference in the formulae arises from the difference in system of allowing for quantity of buttermilk retained in the butter, in the calculation of quantity of buttermilk run off. The Bird & Derby formula has been widely accepted as a basis for calculation of percentage fat loss in buttermilk (7). Wiley (6) has already pointed out that the formula assumes a greater retention of buttermilk in butter than is possible in commercial practice. The discussion and results presented below show that the Bird & Derby formula is based on a false premise. It should be discarded in favour of the Udy formula which can be applied to individual churnings in the same way as the Bird & Derby formula has been applied. The Bird & Derby formula is applicable for churnings of non-washed butter.

Bird & Derby based their calculation of quantity of buttermilk run off on the finding by Mortenson that 'the overrun at time of draining is 20%'. They then derived their formula on the assumption that all the buttermilk in the granules at this stage passes into the butter, but in actual fact a large proportion of the buttermilk held between the granules by capillary action is washed out in the wash-water. The figure for proportion of buttermilk in the granules at time of draining thus is not relevant to the problem at issue. It is patent that the proportion retained cannot be so high as was assumed by Bird & Derby, since this would imply that almost all the water in butter originates from the buttermilk, as shown by the following calculation (see also Wiley (6)):

20% overrun at time of draining $= \frac{20}{120} \times 100 = 16{\cdot}67\%$ of buttermilk in the granules.

Assume total solids content of butter $= 8{\cdot}8$ (S.-N.-F.) $+ 0{\cdot}7$ (fat)
$= 9{\cdot}5\%$,

i.e. water content of buttermilk $= 90{\cdot}5\%$,

and water content of butter at granule stage $= \frac{16{\cdot}67}{100} \times 90{\cdot}5$
$= 15{\cdot}08\%$.

This value is higher than the first moisture tests obtained in many commercial churnings of butter and cannot be correct as an indication of the amount of water retained from the buttermilk, as it does not allow for wash water held among the granules after washing. Udy's figure of 10% of buttermilk in the final butter is of the order of what could reasonably be expected and is in keeping with the normal curd content of butter, but, as stated above, he did not publish the data on which his conclusion was based.

Causes of variation in content of buttermilk retention in butter

The amount of buttermilk retained in the butter cannot be expected to be uniform. The larger the granule churned, the greater is the proportion of buttermilk retained. In addition, for any one size of granule the amount of buttermilk held within the granules in finely dispersed form depends to some extent on the condition of the cream at churning,

on the speed of churning, and on a number of other factors (granules churned rapidly by the early continuous churning processes, and by the more recent Fritz continuous churns, have high buttermilk contents). Vacreator-treated creams give granules which retain the buttermilk in and between them more tenaciously than do flash-pasteurized creams. Granules from cream diluted with water retain more buttermilk than granules from undiluted cream—so much so that churnings of cream containing vat washings must be carefully drained if butter overmoist at the first test is to be avoided. Churning at low temperatures or washing with cold wash-water gives better draining than washing at higher temperatures. The amount of chill-water used in washing affects the dilution of the buttermilk held between the granules, and therefore the quantity of buttermilk retained in the butter when the chill-water is drained; and the proximity of the first moisture test to the limit of 16·0% has an influence on the amount of buttermilk in the finished butter. The ratio of quantity of buttermilk retained to total quantity of butter depends also on the amount of salt added, which affects the total quantity of butter. It is then apparent that any figure for percentage buttermilk retention in butter, to be used in a formula which shall be generally applicable, must be an average of figures showing some considerable variation from churning to churning.

EXPERIMENTAL

Samples of butter and buttermilk were taken from churnings at commercial butter factories in New Zealand during the summer and early autumn months. Most of these factories were using vacreators for treatment of the cream, but two were using flash pasteurizers. No attempt was made to modify the system of manufacture at the factory. The results therefore represent a range of conditions obtaining in general commercial practice in New Zealand.

Methods. Lactose was estimated in the buttermilk, after precipitation of proteins with lead acetate and sodium fluoride, by the Lane and Eynon volumetric method as modified by McDowell (8). The butter (15 g.) was extracted by five successive washings with warm water in a separating funnel as prescribed in the British Standard Method for estimation of lactose in butter (9). The extract was run into a 100 ml. graduated flask. Proteins were precipitated and lactose was estimated as described for buttermilk.

Results. The results are given in Table 1. The percentage of buttermilk in the butter has been calculated from the lactose contents of the buttermilk and butter by the formula

$$\% \text{ buttermilk in butter} = \frac{\% \text{ lactose in butter}}{\% \text{ lactose in buttermilk}} \times 100.$$

The average buttermilk content of butter recorded for the twenty-six churnings at ten factories was 9·4% (range, 7·9–11·5%).

Some estimations of total nitrogen content were made on the butter and the buttermilks from three of the factories. The proportions of residual buttermilk in butter, calculated on the assumption that the nitrogen is present only in the aqueous phase, are shown in Table 2. It is apparent from Table 2 that the ratio of the nitrogen contents of butter and buttermilk cannot be used as a measure of the proportion of buttermilk present in the finished butter. The butters were all made from sweet creams, of original acidity less than 0·15% lactic acid, and neutralized to give a final acidity after pasteurization of 0·07–0·10% lactic acid. The high nitrogen content of the butter was therefore not due to occluded curd particles,

Table 1. *Proportion of buttermilk retained in finished butter, calculated from percentages of lactose in butter and buttermilk*

Factory	System of cream pasteurization	Churning no.	Lactose in butter* (%)	Lactose in buttermilk (%)	Buttermilk content of butter (%)
A	Vacreation	1	0·47	4·47	10·5
		2	0·33	3·55	9·3
B	Vacreation	1	0·37	4·50	8·2
		2	0·40	4·62	8·6
		3	0·38	4·55	8·4
C	Vacreation	1	0·36	4·13	8·7
		2	0·35	4·11	8·5
		3	0·36	3·87	9·3
D	Vacreation with flash pasteurizer preheating	1	0·42	4·46	9·4
		2	0·42	4·50	9·3
		3	0·41	4·46	9·2
		4	0·40	4·11	9·7
		5	0·39	4·11	9·5
		6	0·40	4·09	9·8
E	Vacreation	1	0·39	3·67	10·6
		2	0·43	3·73	11·5
		3	0·37	3·71	10·0
F	Vacreation	1	0·36	3·58	10·1
		2	0·36	3·50	10·3
		3	0·36	3·44	10·5
G	Vacreation	1	0·32	3·19	10·0
		2	0·33	3·20	10·3
H		1	0·33	3·59	9·2
I	Flash pasteurization	1	0·38	4·17	9·1
J	Flash pasteurization	1	0·34	4·28	7·9
		2	0·36	4·27	8·4
				Average	9·4

* The variations in lactose content of the buttermilk were due to variations in farm and factory dilution of the cream, and not to use of break water.

Table 2. *Total nitrogen contents of butter and buttermilk, and percentage of total nitrogen in butter as a percentage of the total nitrogen in buttermilk*

Factory	Churning no.	Total N in butter	Total N in buttermilk	$\frac{\%\text{ N in butter}}{\%\text{ N in buttermilk}} \times 100$
A	1	0·080	0·53	15·2
	2	0·088	0·49	17·8
	3	0·085	0·49	21·5
B	1	0·088	0·50	17·5
	2	0·088	0·50	17·5
	3	0·083	0·50	16·6
C	1	0·094	0·43	22·1
	2	0·080	0·42	20·4
	3	0·086	0·42	20·4

but rather to the nitrogen present in the phospholipid and other protein constituents of the fat-globule adsorption layer. Lactose as a crystalloid is likely to be evenly distributed throughout the aqueous constituents of the buttermilk and the butter, except for the minor effect of some bound water present on the protein (McDowall, Dolby & McDowell (10)).

In view of the possible range of variation in extent of buttermilk retention in butter, due to the causes set out earlier, and of the possibility of some slight loss of lactose to the wash water by diffusion from the granules, the average value 9·4 % found from the figures

in Table 1 is sufficiently close to the value, 10%, adopted by Udy to justify accepting the Udy formula as of general applicability. It can be applied to a churning of butter from cream diluted with water in the same way as the Bird & Derby formula has been applied, if F and f are taken as the fat contents of the cream *as churned* and of the buttermilk *actually run off*, respectively.

Non-washing of butter and fat loss in buttermilk. When the washing of butter with chill-water is omitted, the degree of buttermilk retention in butter is higher because the buttermilk between the granules at time of draining is worked into the butter. This gives a corresponding rise in curd content of the butter. If the first moisture test is low and water is added to make up the moisture content to the final value, the buttermilk retention will be lower than when a high first moisture test is run. In general, however, a first moisture test of 15·0–15·4% may be taken as normal for good commercial churning practice on vacreator-treated cream. The value 15·08% for percentage in the final butter of moisture originating from the buttermilk, as derived above from Bird & Derby's premise, is thus representative of conditions when the butter is not washed, and the Bird & Derby formula is applicable for calculation of percentage losses of butterfat in buttermilk from non-washed churnings of butter.

Conclusion. The factors for calculation of percentage fat loss from creams of different fat contents, for washed butter (Udy's formula) and for non-washed butter (Bird & Derby's formula) are given in Table 3.

Table 3. *Factors for calculation of percentage fat loss in buttermilk from the buttermilk fat test, for creams of fat contents in the range* 20–49%

To obtain the truly correct value, multiply result by $\frac{100}{100-f}$ (where f is fat test of buttermilk).

Fat content of cream (%)	Factor for		Fat content of cream (%)	Factor for	
	Washed butter	Non-washed butter		Washed butter	Non-washed butter
20	3·88	3·80	35	1·73	1·66
21	3·64	3·56	36	1·65	1·58
22	3·43	3·35	37	1·58	1·50
23	3·23	3·15	38	1·51	1·43
24	3·05	2·97	39	1·44	1·36
25	2·88	2·80	40	1·38	1·30
26	2·73	2·50	42	1·26	1·18
28	2·45	2·37	43	1·20	1·13
29	2·33	2·25	44	1·15	1·07
30	2·21	2·13	45	1·10	1·02
31	2·11	2·03	46	1·05	0·97
32	2·01	1·92	47	1·01	0·93
33	1·91	1·85	48	0·96	0·88
34	1·81	1·74	49	0·92	0·84

SUMMARY

The Bird & Derby method of calculating percentage butterfat loss in buttermilk is in error because it assumes that all the buttermilk in the granules at draining is retained in the buttermilk, whereas much of it is washed out in the wash water. Lactose estimations in butter and buttermilk confirm Udy's finding that butter contains about 10% of buttermilk. The Udy formula, viz.

$$\% \text{ fat loss} = \frac{(100 - 1{\cdot}12F)}{F} \times f,$$

is therefore in this respect correctly based and can be applied to individual churnings of butter in the same way as the Bird & Derby formula has been applied. The Bird & Derby formula should be discarded except for use when the granules are not washed with chill-water.

Both the Udy and the Bird & Derby formulae do not allow for the content of fat in the buttermilk retained in the butter or for the influence of fat content of the buttermilk on the calculated quantity of serum lost. When these factors are taken into account the Udy formula becomes

$$\% \text{ fat loss} = \frac{100}{100-f} \times \frac{(100 - 1{\cdot}12F + 0{\cdot}0012fF)}{F}.$$

The correction $+0{\cdot}0012fF$ is not significant.

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391. THE EFFECT OF PENICILLIN IN MILK ON THE MANUFACTURE OF CHEDDAR CHEESE

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The lactic streptococci employed as starters for the manufacture of cheddar cheese in New Zealand have recently been shown to be sensitive to the action of penicillin (1). The purpose of this paper is to report results of investigations undertaken to ascertain the effect of penicillin in milk on the manufacture and ripening of cheese under commercial conditions.

ADDITION OF PENICILLIN TO THE CHEESE MILK

Penicillin solutions in sterile distilled water were prepared from the dry calcium salt (commercial preparation available for veterinary use), and assayed daily by the filter paper-disk method (2) with *Staphylococcus aureus* Oxford strain H as test organism. Since the desired concentrations of penicillin in the cheese milk (0·03–0·20 units/ml.) were too small to be estimated accurately, an intermediate dilution was made by adding an appropriate amount of aqueous penicillin solution to 10 lb. of milk. A small portion of this milk was retained for assay of the penicillin, and the remainder was added to the 500 lb. of milk to be made into cheese. Penicillin in the intermediate milk dilution was assayed by an adaptation of the paper-disk method used in the Veterinary Pathology Department, Massey College.

METHOD OF CHEESE MANUFACTURE

For the main experiments, 500 lb. batches of mixed milk of good quality were flash pasteurized at 155° F. and passed over an open vertical cooler before being run into 80-gallon vats. Vat 1 served as a control. The experimental vats contained milk to which penicillin had been added just prior to pasteurization. The cheese-making procedure in all the vats was similar, but the successive steps in manufacture were based on acidity. Thus the curds were 'dried' at 0·16%, 'milled' at 0·65%, and 'salted' at 0·80–0·84% acidity. It was then possible to gauge the effect of added penicillin on the development of acidity by the length of time taken by the different curds to reach these acidity values. The cheeses were ripened in a curing room at 55° F. for 14 days, and held thereafter at 45–50° F.

THE EFFECT OF PENICILLIN IN CHEESE MILK ON STARTER HP

The effect of various concentrations of penicillin in the cheese milk was first determined using strain HP as starter. The penicillin was added to the milk before pasteurization at 155° F. An example of the manufacturing details is given in Table 1.

It will be seen that acid production by culture HP is influenced by the presence of penicillin in the cheese milk in concentrations above 0·07 unit/ml. The bacteriostatic action of penicillin in a concentration of 0·10 unit/ml. in the cheese milk pasteurized at 155° F. appeared to be relatively constant against this starter. The vats were 20–30 min. slower than the controls. A concentration of 0·15 unit/ml. resulted in a vat 'failure' at about 'milling' time.

Table 1. *Manufacturing details*

500 lb. milk, 1¾% starter HP, 5 min. 'ripening', 'setting' temperature 88° F., 'cooking' temperature 98° F. Acidity at 'drying' 0·16%, acidity at 'salting' 0·80–0·84%.

Vat	Concentration of penicillin in cheese milk (unit/ml.)	Time (set–dry) hr.	min.	Time (dry–salt) hr.	min.	Time (set–salt) hr.	min.	Difference total time hr.	min.
1	0·00	2	35	2	30	5	5	0	5
2	0·07	2	38	2	32	5	10		
1	0·00	2	40	2	35	5	15	0	25
2	0·10	2	40	3	0	5	40		
1	0·00	2	35	2	45	5	20	0	55
2	0·12	2	40	3	35	6	15		
1	0·00	2	40	2	35	5	15	0	55
2	0·13	2	40	3	30	6	10		
1	0·00	2	35	2	30	5	5		
2	0·15	2	44	(4	0)	Acid production ceased at 0·64%			

COMPARISON OF THE SENSITIVITY OF VARIOUS STARTER CULTURES

A comparison of the sensitivity of single-strain cultures at the 0·10 unit/ml. level was made under similar conditions. Some of the stock strains of starter streptococci are more active than others, and it is the practice in commercial factories to increase the percentage of the slower starters used in the vats, in order to allow for this difference and to obtain regularity of acid development from day to day. The percentage of starter used throughout these experiments was varied accordingly. Actually, the strict regularity in acid production desired was not achieved in these particular trials, but the performance of the starters was sufficiently close to allow comparisons to be made. The results are shown in Table 2.

Table 2. *Comparison of effect of penicillin on starter cultures*

Starter	%	Vat	Concentration of penicillin (unit/ml.)	Time (set–dry) hr.	min.	Time (dry–salt) hr.	min.	Time (set–salt) hr.	min.	Difference total time hr.	min.
K	2¼	1	0·00	2	55	2	30	5	25		
		2	0·07	3	00	(3	30)	Acid production ceased at 0·38%			
R_1	2	1	0·00	2	50	2	30	5	20		
		2	0·10	2	57	(3	55)	Acid production ceased at 0·67%			
R_6	1¾	1	0·00	2	38	2	12	4	50	0	40
		2	0·10	2	42	2	48	5	30		
E_8	2¼	1	0·00	2	35	3	20	5	55	0	20
		2	0·10	2	40	3	35	6	15		
ML_1	2¼	1	0·00	2	45	3	20	6	5		
		2	0·10	2	50	(4	20)	Acid production ceased at 0·74%			
ML_3	1¾	1	0·00	2	30	2	35	5	5	0	15
		2	0·10	2	35	2	45	5	20		
		3	0·00	2	22	2	28	4	50	0	50
		4	0·17	2	25	3	15	5	40		

The figures show that culture K was the most sensitive starter, 0·07 penicillin unit/ml. being sufficient to stop acid production soon after 'drying'. Cultures R_6 and E_8 reacted to a concentration of 0·10 unit/ml. in a somewhat similar manner to culture HP, i.e. the vats were 20–40 min. slow. At this level cultures R_1 and ML_1 ceased to produce acid towards 'milling' time. All these cultures are pure strains of *Str. cremoris* (Orla-Jensen). Culture

ML_3, a *Str. lactis* type, was more resistant. The highest concentration of penicillin employed (0·17 unit/ml.) did not cause a complete failure with this starter.

OBSERVATIONS ON MANUFACTURE AND GRADING OF CHEESE MADE FROM PENICILLIN-TREATED MILK

When penicillin was present in the milk in such concentrations as to affect the rate of acid production of the starter, the curds tended to be 'short' and 'chippy', varying to 'harsh' with the larger amounts of penicillin.

(1) *Cheese manufactured with HP starter*

At 14 days, the cheese made from the control milks were of good quality with smooth body and clean mild flavour. Cheese from milk containing 0·07–0·10 penicillin unit/ml. were slightly 'sweet'. 0·15 unit/ml. resulted in 'sweet', 'curdy' second-grade cheese. At levels between 0·10 and 0·13 unit/ml. the cheese were 'weak', 'sweet' and 'lumpy'. The flavour was normal in all the experimental cheese.

At 3 months, the control cheese were characterized by a good smooth waxy body and a normal 'cheesey' flavour. 0·10 penicillin unit/ml. in the cheese milk gave cheese which were a shade 'pasty' in body. The flavours varied from normal to slightly 'off' or slightly 'rancid'. Above this level the cheese were 'weak' and 'sticky' with slightly 'off', 'rancid' and 'fermented' flavours. 0·15 unit/ml. gave a very 'weak', 'pasty' and 'fermented' cheese.

(2) *Cheese manufactured with various starter cultures*

The cheese from milk to which penicillin had been added were poorer in quality than the controls at 14 days. When acid production stopped, the cheese were of second-grade quality. At 3 months, cheese from vats which were 30–40 min. slow were 'weak' in body and the flavours were 'off'. 'Dead' vats gave 'weak', 'fermented' cheese.

On the whole, the defects in the final cheese could be correlated with the adverse effect of penicillin on the rate and extent of acid development by the starter cultures in the vat during manufacture.

THE EFFECT OF HEAT TREATMENT ON THE ACTIVITY OF PENICILLIN IN MILK

Comparisons of the effect of penicillin added before and after pasteurization of the cheese milk, and comparisons of flash pasteurization of milk containing penicillin at 155° F. with flash pasteurization at 160° F., showed that in general the differences in results were almost negligible.

It is clear that pasteurization within the temperature limits available to the cheese-maker does not appreciably alter the activity of penicillin in milk. It has been noted that penicillin retains a portion of its original potency after heat treatment at higher temperatures. Tests on twenty milk samples from penicillin-treated udder quarters confirmed previous findings(1).

The samples which were collected from cows at different milkings and which contained penicillin concentrations of 1·3–4·5 units/ml. were steamed for a period of 1 hr. A loss in potency averaging 52% (range 45–59%) resulted. Similar samples autoclaved at 10 lb. pressure for 20 min. showed an average loss in activity of 67% (range 56–76%). Autoclaving at 15 lb. for 15 min. caused a deterioration of 73% (range 70–77%). It may be

remarked that the variation in degree of deterioration is somewhat wide. Apart from slight differences in conditions of heat treatment from batch to batch, or possible variations in milk composition, it is worthy of note that the milk from quarters treated with the calcium salt of penicillin all fell within the lower portions of the range, and milks containing the sodium salt showed the greater deterioration on heating.

As a matter of interest, further experiments were carried out in which penicillin calcium at a concentration of 4 units/ml. in milk (pH 6·6), in sterile water (pH 7·0), and in sterile phosphate buffer (pH 7·1) was steamed and autoclaved for purposes of comparison. The resultant losses in strength after steaming 1 hr., autoclaving at 10 lb. 20 minutes, and 15 lb. 15 minutes respectively were as follows: (1) in milk, 45, 55, 64%; (2) in water, 99–100% for all three treatments; (3) in buffer, 98–99% for all three treatments. It seems evident, therefore, that penicillin is more heat stable in the presence of milk than it is in aqueous solutions.

THE AMOUNT OF PENICILLIN IN MILK FROM TREATED UDDER QUARTERS

The practical dairyman's interest as far as the influence of penicillin in cheese-making is concerned is centred on the question of how many cows (or how many udder quarters) in a herd can be penicillin-treated before trouble can be expected in the cheese vat. (This is assuming that the milk produced during and immediately after treatment is included in the main bulk.) The question obviously cannot be answered precisely, because cows vary in the volume of milk they secrete, and in the excretion of penicillin after treatment. But some approximate answer may be given on the basis of experiments carried out with cows in the Massey College herd. Six cows were selected for the investigation, all being sub-clinical cases of mastitis. The milk from the quarters showed only a few small clots in the first milk drawn and would be considered normal by the average dairy farmer. Only one udder quarter of each cow was infected and received penicillin treatment. Two of the animals were given an infusion of 17,000–20,000 units of penicillin in 100 ml. sterile distilled water (per infected quarter) at five successive milkings. The other four animals were given four infusions of the same strength at 24 hr. intervals. All the treated quarters were milked separately and completely throughout the period of the investigation, and assays for penicillin content were carried out by the paper-disk method on the total milk drawn from each treated quarter at each milking. The results summarized in Table 3 are those obtained from assays of the milk samples taken at milkings following a treatment, e.g. if an infusion was given after the evening milking, the sample was taken at the morning milking. The highest concentration of penicillin found was 5·0 units/ml. and the lowest 1·3 units/ml. The total number of units of penicillin in the milk ranged from 1000 to 10,580, and the amount excreted in the milk was relatively constant for each quarter at different milkings. There was no substantial 'build up' of concentration of penicillin in the udder as a result of successive treatments.

From these results (which are admittedly few in number) the proportion of quarters under penicillin treatment which would contribute sufficient milk into the general bulk to affect a cheese curd adversely may be estimated. At the critical level of 0·10 unit/ml. in milk, which is sufficient to check most single strain starter cultures, approximately 405,000 units of penicillin would be necessary in a 900 gallon vat of milk. On the basis of the lowest value obtained from the twenty-six results from six quarters tested it would take milk from 405 treated quarters to affect acidity production in the vat; on the basis of the

highest value it would take thirty-eight quarters. As would be expected, the possible variation is wide and might be even wider if a larger number of cows were examined. Safety measures in practice would have to be based, however, on the minimum number of treated quarters which might cause trouble, and it is evident that rejection of the milk from cows undergoing treatment with penicillin is the only reasonable safeguard so far as cheese manufacture is concerned.

Information on the time taken for penicillin to leave the udder was obtained from assays of the milk samples collected after treatment finished. At the second milking following the cessation of treatment, i.e. 24 hr. after the last infusion, the concentration of penicillin found in the milk varied from about 0·10 to 0·5 unit/ml. At the next milking no penicillin could be demonstrated by the paper-disk method. It would seem unnecessary, therefore, to exclude milk from treated quarters from the factory supply beyond two milkings.

Table 3. *Penicillin concentrations in milk after udder infusion*

Cow-quarter	Breed	Daily milk yield before treatment (lb.)	Stage of lactation (days)	Treatment: units of penicillin administered (frequency)		Penicillin concentration (units/ml. in total milk from treated quarter per milking)	Milk weight per treated quarter (lb.)	Penicillin units excreted	No. of quarters to provide critical penicillin level in 900 gallon vat
Ze	Jersey	18	122	17,000 units at 5 successive milkings	Average	3·1	1·7	2372	171
					Range	2·0–3·8	0·6–2·3	1000–3105	405–130
Td	Jersey	45	11	17,000 units at 5 successive milkings	Average	3·5	5·7	8978	45
					Range	2·4–5·0	4·7–6·2	6696–10,580	60–38
Sd	Friesian	22	266	4 treatments 20,000 units at 24 hr. intervals	Average	4·4	3·5	6930	58
					Range	4·3–4·5	3·4–3·5	6580–7088	62–57
Ua	Friesian	72	31	4 treatments 20,000 units at 24 hr. intervals	Average	1·5	6·9	4658	87
					Range	1·3–1·6	6·5–7·5	4388–4680	92–87
Jd	Jersey	28	109	4 treatments 16,000 units at 24 hr. intervals	Average	2·4	3·5	3780	107
					Range	2·2–2·6	3·2–3·6	3695–4407	110–92
Pd	Jersey	26	138	4 treatments 16,000 units at 24 hr. intervals	Average	2·8	2·9	3655	111
					Range	2·3–3·0	2·8–3·0	3384–4111	120–98

The efficacy of penicillin in the treatment of mastitis in dairy herds is undoubted, and its use is likely to be more widespread in the future. There is a tendency to adopt a 'blitz' method of treatment on herds in which there is a high incidence of mastitis in order to prevent spread of infection. It is conceivable, therefore, that milk delivered to a cheese factory may, on occasions, contain a relatively high proportion of milk from treated quarters. That sufficient penicillin to affect the starter may gain access to the cheese vat is evident from experiences at the Institute factory. The erratic behaviour of experimental vats during one week was traced to the accidental inclusion of milk from treated quarters in the factory supply. One vat contained 1600 lb. of milk, 1200 lb. of which came from one milking shed or dairy, and contained the night's (four quarters treated) and the morning's (five quarters treated) milk from two cows undergoing treatment for mastitis. The starter used was the single strain R_6 and the vat was slow to 'drying' and very slow to 'milling'. On another occasion 35 lb. of milk from eight quarters at different stages of penicillin

treatment was deliberately added to 1206 lb. of normal cheese milk. Another vat contained only normal milk and served as a control. With HP as starter, the experimental vat was 10 min. slower to 'drying', 40 min. slower to 'milling', and 50 min. slower to 'salting' than the control. Computation based on these two cases, shows that fifty to sixty quarters treated at one milking could affect the starter in a 900 gallon vat of milk.

Apart from these experiences, very strong presumptive evidence that slow vats have been caused by the presence of penicillin in the cheese milk was reported by Mr J. J. Walker, Dairy Instructor, Taranaki. At the beginning of the season, a large number of cows were treated amongst herds supplying a particular cheese factory. The demand for penicillin became so great that farmers were instructed in the method of treatment so that they themselves could administer penicillin to the cows. During this period, the behaviour of the starter cultures in the cheese factory became very erratic and acid development in the vats showed a marked tendency to 'drag'. No improvement was observed on increasing the percentage of starter, or on replacing the cultures with fresh ones from the laboratory. Some poor quality cheese was made as a result of the abnormal conditions. When it was realized that starter cultures were sensitive to the action of penicillin, the farmers were requested not to deliver milk from treated quarters for at least two milkings following treatment. Acid development in the cheese curd immediately became more rapid and cheese manufacture became normal. It is not possible to state definitely that penicillin was the cause of the slow vats, but there is evidence to show that a very large number of cows were being treated at this particular period. The sequence of events suggests that the extensive use of penicillin was probably the factor responsible for slow acid development in the cheese vats.

DISCUSSION

The effect produced by the smaller doses of penicillin on the rate of acid development in the cheese milk is very similar to the 'slowness' brought about by a light infection of the milk with bacteriophage (3). The vats are not noticeably slow until the later stages of manufacture, and in commercial practice it would be difficult to ascertain whether penicillin was the cause of the erratic performance of the starter. Possibly, organisms growing in the presence of a bacteriostatic agent may become more susceptible to the influence of phage action and thus 'slowness' may be due to a combination of factors. No readily applied test is available for detecting the presence of such small concentrations of penicillin as will interfere with the normal growth of starter cultures in milk. Penicillin can thus be implicated as a cause of slow vats and 'sweet' cheese by circumstantial evidence only.

The aim of the cheese-maker is to produce cheddar cheese of the finest quality possible. To do this he must pay special attention to the performance of the starter and to the elimination of the causes of slow acid development during the cheese-making process. Experience in the field has shown that sufficient penicillin to influence the behaviour of the starter may sometimes be delivered to the cheese vat with consequent effect upon cheese quality. In some cases, however, a spasmodic or mild influence may not be readily recognized during manufacture, but yet may cause a lowering in quality of the resultant cheese.

The problem is complicated by the fact that different methods for treatment of mastitis with penicillin are available. Udder infusion appears to be the only efficient way of bringing the antibiotic in contact with the infecting organisms. Differences of opinion are expressed by various workers as to the optimum doses and frequency of administration. The doses

cited in literature vary from 10,000 to 200,000 units per quarter for one to five treatments given at successive milkings or at intervals ranging from 12 hr. to 3 days. These circumstances, as well as the experimental work quoted in this paper showing the wide variations encountered in the amount of penicillin in milk drawn from treated quarters, make it impossible to decide on a safety margin in the number of cows which could be treated in herds supplying cheese factories without affecting the performance of the starters or the final quality of the cheese. Viewing the position as a whole, it would appear that, although the possibility of penicillin being responsible for 'dead' vats may be remote, its presence in the milk is always a potential danger, and the evidence available is sufficient to merit caution in its use. This does not mean that measures should be taken to prevent the use of penicillin to its best advantage, but rather that the farmer should be acquainted with the possible dangers involved in supplying milk containing penicillin to the cheese factory. The rejection of milk from quarters undergoing treatment is a logical precaution.

SUMMARY

Penicillin present in cheese milk affected the performance of various 'single strain' starter cultures in the cheese vat.

A concentration of 0·07 penicillin unit/ml. in the milk was sufficient to cause a complete failure or 'dead' vat with the most sensitive strain of *Str. cremoris*. Two strains ceased to produce acid in the later stages of the manufacturing process when the milk contained 0·10 unit/ml. With three other strains the 0·10 unit/ml. level delayed acid development; the experimental vats required 20–40 min. longer than the controls to reach 'salting' acidity.

Flash pasteurization temperatures usually employed in cheese-making (150–160° F.) did not appreciably alter the activity of penicillin in the cheese milk.

The quality of the cheese produced was influenced by the rate and extent of acid production by the starter cultures in the vat during manufacture.

Experience in the field indicates that where a large number of cows are treated simultaneously for mastitis, there may be sufficient penicillin in the cheese milk to affect the starter, if the milk from quarters undergoing treatment is included in the bulk milk.

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REVIEWS OF THE PROGRESS OF DAIRY SCIENCE

SECTION E. DISEASES OF DAIRY CATTLE

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The period covered by the review extends from about the beginning of 1942 to the end of 1948. Within the limits of the available space it is impossible to deal adequately with all the many papers on these three diseases which have appeared during this period, and it has therefore been necessary to confine attention to what appears at present to be the more profitable lines of research, though obviously this has meant omitting much which may later become important.

I. MASTITIS

A. *General*

The contention of early workers that *Streptococcus agalactiae* is the cause of most mastitis and that it could be eradicated by regular bacteriological testing with segregation and disposal of infected animals has been reaffirmed by the recent publication of Palmer(1) who states that 80–90% of all mastitis is due to this organism, and of Cunningham, Wilson, Ineson, Raymont & Mannon(2) who report nine out of thirteen herds completely cleared of this disease. They state that the rate of herd clearance depends on the proportion of infected cows in the herd, the number of infections which develop after eradication begins and the average period infected cows are kept in the herd. They emphasize that *Str. agalactiae* is the major cause of clinical mastitis and state that the incidence of blind quarters was reduced by 70% after the eradication of this organism. Elimination of *Str. agalactiae* infection did not cause any increase in clinical disease due to *Str. dysgalactiae*, *Str. uberis* or staphylococci. They did not encounter reintroduction of the disease into the herds through infected heifers, and report that 574 heifers in herds where mastitis milk was fed to the calves were all clear of infection up to 3 months after calving. Another group of 350 heifers used to form six herds were all clear of infection and the herds so formed have remained clear. (Miller & Heishman(3) record one infected heifer out of seventy-seven, of which forty-five were from heavily infected farms.) On the other hand, Watts(4) was disappointed in the results obtained in a considerable field trial of this method. Of sixty-five herds with some 5000 cows only four were freed of infection. He attributes this bad result partly to the use of unsatisfactory milking machines. He showed that where such a machine was changed to a satisfactory type an immediate decrease occurred in the infection rate of contagious mastitis. This effect of changing the machine has been confirmed by Udall(5) who found in one severely affected herd that until the machine was changed no remedial measures affected the mastitis infection rate. Stevenson(6) attributes such results to the effect of injury to the teat, bringing evidence that the reduction of the vacuum on the machine reduced the incidence of the disease. That milking practices affect the udder and thus the incidence rate is also the view of Parkin(7) and Moss(8). The latter states that in Honolulu irregular milking followed the Pearl Harbour disaster and cows in herds previously little affected by mastitis showed considerable clinical disease. Little & Plastridge(9) report similar clinical disease in an agalactiae-free herd when the milking machine was changed. They related this to the use of too high a vacuum. A similar finding was recorded by Neave, Sloan & Mattick(10), where the vacuum gauge recorded 15 in. while the actual vacuum was 19½. In this *Str. agalactiae*-free herd of sixty cows twenty-six developed clinical symptoms mainly due to *Str.*

uberis which stopped when the vacuum was reduced. According to Ward, Whittleston, Hopkirk & Filmer(11), one milking practice—that of hand stripping after machine milking—can be omitted without causing mastitis in herds where it has been a routine milking measure.

These reports are compatible with Murphy's(12) theory that mastitis infection should be regarded as threefold: invasion, infection, inflammation; rather than twofold: infection, inflammation. The concept that in ordinary herd invasion, the mere presence of the organism on or in the teat could not be avoided and that control should aim at preventing 'infection' was inherent in much previous work by other authors, but Murphy is thought to be the first to formulate a definite 'theory'.

That 'invasion' in Murphy's sense of the term is constantly occurring in all milking cows was shown by the British group of workers on this disease(13) and by Ingalls & Johnson(14). In the British work nine herds were sampled at weekly intervals for 6 months. In all 16,482 samples of milk and 5433 swabs from the skin of the teats were examined. The proportion of cows found infected at least once during the 6 months varied in the different herds from 35·6 to 100% when milk samples were considered and from 45·7 to 100% when the teat samples were taken as the criterion of infection. Some of the cows are reported to have been persistent skin carriers (often but not always associated with sores on the teats) though not infected in the milk. That teat infection rates of 100% are not unusual in practice is borne out by Watts's(15) figures for those farms where samples taken after different methods of washing the cows gave infection rates varying from 32 to 278% greater than the correct infection rates as judged by sampling the cows with precautions against adventitious contamination. Whether the high rate of teat infection is due to repeated reinfection by hands, bedding, etc., is not certain, but Spencer, McCarter & Beach(16) point out that if the organism can survive for long periods in bedding the methods of control would need to be altered. They therefore carried out experiments in which varying numbers of *Str. agalactiae* were added to barn bedding and bovine faeces to determine the minimum numbers which could be recovered from these heavily contaminated materials. They used direct plating of dilutions on selective media and found that they could recover the organisms when approximately 500 were present per g. of bedding and 100,000 per g. of faeces. Four attempts to recover *Str. agalactiae* from bedding under infected cows were unsuccessful, nor were swabs from the actual byre floors positive. They did, however, recover it from the hands of two hand milkers but not from those of eight men milking by machine. They also added *Str. agalaciae* cultures to sterile bedding under laboratory conditions and found that death was rapid, the majority of any culture being dead within 24 hr. though colonies were obtained up to 9 days.

Cunningham, Stirling, Raymont & Wilson(17) consider that contamination is the major factor causing discrepancies in successive tests of a herd. They state that it is not possible to confirm 'plating negative'-'enrichment positive' infections by repeated plating, but in one herd, where more stringent precautions were taken against contamination, they demonstrated complete agreement between the usual direct plating technique and enrichment methods. They describe their method of sampling in detail, an interesting feature being their use of a special leather harness to hold the sampling bottles. This harness also carries a light which is of great value in badly lit cowsheds during winter. They take great precautions in washing and sampling the cows to prevent contamination and consider that any *Str. agalactiae* present on the plate indicates probable udder infection. That such precautions are not impossible under practical farm conditions was shown by Watts(15), who introduced an infected cow into a free herd where the cows were washed with water. Teat swabs taken during the succeeding 3 weeks showed teat infections of 22·2, 87·5 and 45·8% respectively. Hypochlorite 1000 p.p.m. was then added to the washing water and a separate cloth used for each cow. The infection rates on the teats for the next 3 weeks were 0, 0 and 8·3%, although the infected animal remained in the herd. The use of hypochlorite was then discontinued and the teat infection rate rose again.

Animals which are either themselves persistent teat carriers or are constantly being recontaminated on the outside of the teats would presumably only become infected within the udder if some factor or factors made them more susceptible to the organism or rendered the teat sinus

particularly accessible. Both these causes would be operating in the case of overt injury to the teat but Ferguson (18), who points out the ease with which injured teats are infected when artificially exposed to infection, found that field observations did not confirm that *Str. agalactiae* infection commonly followed udder or teat injury. Of 283 quarters infected by bacteria following trauma, *Str. agalactiae* was isolated from only 24%, *Str. dysgalactiae* from 23%, *Str. uberis* from 15%, other streptococci from 11% and staphylococci from 13%. Unless her results were obtained from abnormal herds this evidence would seem to show that while obvious traumatic injury may cause bacterial infection the proportion of such infection due to *Str. agalactiae* is not comparable to the extent of infection by this organism as found by general field examinations (24% as compared to 80%). Thus this particular factor cannot be the only or even the major one in the spread of *Str. agalactiae* mastitis in the field.

Nevertheless, Sanders (19) suggests that the spread of mastitis may be aided by the damage done to the teats by the horn-fly *Lyperosia irritans* which leads to deformed teat sphincters and thus to 'patent' teats. That this type of teat is a cause of mastitis is claimed by Murphy (20) who estimated 'patency' by using his thumb and forefinger to apply sudden lateral pressure to the teat when full of milk. Of 205 quarters so examined he considered seventy-seven to be 'patent', and he records 50·7% of these infected with either streptococci or staphylococci. Of the remaining 128, 32·1% were infected. Further work on eighty-seven cows repeatedly tested showed that 'patency' as judged by this method was not constant but, nevertheless, he considers that the differences in incidence rate recorded are significant.

He does not consider that teat patency is associated with the well-known 'increasing with age' pattern of streptococcal mastitis, as in a further article he suggests that this pattern is a function of age itself, i.e. it is the age of the cow which predisposes her to infection. This suggestion is based on his observations on the natural disease in two herds of different breeds kept under similar conditions. In the 6-year period one-third of the cows did not become infected at all, and the remainder were free for the first two lactation periods. Following this free period the age-infection ratio was a straight line and from this he deduces that infection is due to some change in the body of the cow as she ages. Murphy, in collaboration with Pfau, Lepard & Bartlett (21), has also studied the possible influence of hereditary factors. They compared the mastitis histories of two families consisting of six and seven cows and found the first to be infected with streptococci during 24% and with staphylococci during 39·3% of their total milking lives, while the figures for the other family were 0·9 and 10·9% respectively. They conclude that heredity definitely affects the incidence of mastitis. While this may be true, their figures seem rather few on which to base a definite assertion. Murphy (22) considers that this hereditary factor is not associated with any outstanding irregularity (e.g. pouches) in the lining membrane. He considers these irregularities to be a breed or individual characteristic.

Whether non-specific mastitis (used here to refer to apparent non-bacterial mastitis) is a predisposing factor or whether previous infection with other organisms such as staphylococci predisposes to infection with *Str. agalactiae* is not decided.

Hastings, Beach & Johnson (23) found that *Bact. aerogenes* was frequently present in milk, but they could not correlate its presence with changes in the milk (see however Burkhardt, Beach & Spencer (24)), or with the degree of infection with *Str. agalactiae*. Crawshaw (25) suggests that one of the primary factors in inducing mastitis is the exposure to cold of animals so housed and managed that they are particularly susceptible. He states that if cows are kept from birth onwards under severe climatic conditions they are acclimatized to cold and have very thick winter coats. He claims to have produced a strain of Jersey cows immune to 'catarrhal mastitis'. By this term he means a non-infective condition characterized by the presence of mucus in the milk which he believes is incurable and frequently the precursor of actual infection. These observations may be compared with those of Wilson & Cunningham (26) who believe that their observed high incidence of staphylococcal infections in suckled cows was due to the majority of the animals of this type being kept in the open air or in courts. They state that court and open-air systems are associated with about twice the incidence of staphylococcal infections as compared

with animals kept in stalls whether the latter are milked or suckled. Malcolm, King & Campbell (27) investigated twenty-eight heifers in a herd of 100 animals by examining milk samples from individual quarters at weekly or fortnightly intervals throughout their first lactation. Leucocyte counts, electrical conductivity tests and examination for specific pathogens were carried out. Twelve remained normal, four showed various abnormal conditions of the udder due to non-specific causes and twelve showed mastitis (i.e. rise in cell count and increased conductivity). In five, staphylococci were isolated sufficiently numerous to indicate infection, while in a further five no bacteria could be incriminated. (Unfortunately the authors record some evidence that *Brucella abortus* infection was present in the herd but they were not able to examine the samples for this organism.) There was no evidence that the type of mastitis they record predisposes the animals to infection with *Str. agalactiae*, since, although this organism was present in the main herd, none of the heifers became infected. Murnane (28) gives a short review of what he considers the predisposing factors in mastitis, in which he includes the stage of lactation stating that cows are most susceptible at the beginning and end of lactation.

While mastitis is more apparent in milking herds it does occur in beef herds where the cows are suckled, e.g. Munch-Petersen (29). In these cases no obvious predisposing factors occur and since the animals are in a natural habitat the infection is light and not epidemic in character.

B. *Diagnosis*

Many methods of diagnosis have been detailed ranging from the examination of the bulk milk of the herd to bacteriological examination of each quarter of every cow. Johnson & Bryan (30) concluded that the microscopic examination of incubated milk from the bulked milk of the herd was sufficient to show if mastitis was present. They found that in eight-three herds sixty were correctly diagnosed as infected and fourteen as non-infected. In four herds positive findings were not confirmed on individual cow examinations, while in five negative findings were incorrect. Mastitis in individual cows can be diagnosed in the actual cowshed by physical examination (Murphy, Udall, Johnson & Tompkins (31) and Andberg & Weirether (32)). The article by Murphy *et al.* is based on the physical, bacteriological and biochemical examination of 120 cows in a tuberculosis- and abortion-free herd. The udders were placed in four classes depending on the degree of physical change. It was shown that streptococci were absent from the few completely normal udders in Class 1 and present in 80% of the class 4 udders (those showing most induration). Correlation between physical examination and bacterial infection was not good in classes 2 and 3 in which the majority of the cows were placed. The 1 hr. resazurin test (conducted in the shed) has been advocated by Morgan & McKenzie (33), though these authors do not claim great accuracy for this method. Roach (34), after comparing the indicator, strip cup, chloride, catalase and Hotis tests together and with physical examination and leucocyte count, considers that indirect tests either in the shed or in the laboratory are of very limited value owing to the high proportion of false negatives if only one test is used and of false positives if a number are employed. He considers the most reliable test to be the strip-cup test if used repeatedly. The leucocyte count has been further examined by Hucker (35) who found that 68% of 30,331 fore-milk samples (quarters) of 8000 cows contained less than 100,000 leucocytes per ml., 20% contained more than 500,000 and 10% more than 1,000,000. Although he considers any quarter showing more than 500,000 to be infected, he does not advocate that any count less than 1,000,000 should be considered definitely positive. He points out that on the average machine-milked herds tend to have higher leucocyte counts than hand-milked. Cone (36) agrees with this and also asserts that normal individual variations in both the leucocyte count and the chloride content is often greater than the changes induced by infection.

Another indirect test is the fluorescence given by thin layers of milk when irradiated by ultra-violet light. Schönberg (37) states that while normal milk fluoresces brilliantly 'mastitis' milk nearly always gives dull colours.

The original Hotis test has been compared with direct plating of samples by several authors.

Biddle (38) criticizes it, stating that the plate method is more accurate and equally rapid. Stirling & Watts (39) also found that the test was not sufficient in itself but that, if any culture showing a yellow colour was plated, a high degree of correlation (above 90%) with the plate test was obtained, with an 80% saving of time and materials. They used the test applied to composite cow samples and compared it with the plate test of quarter samples. Miller (40) also reports 90% correlation between the Hotis test and blood-agar plates in a series of 10–15,000 examinations, while Schalm (41) found that if all positive Hotis samples were examined microscopically 87% of correct results were obtained (4132 samples). He distinguishes seven types of Hotis reaction:

(1) Negative	Grey
(2) Suspicious	Grey to purple with zones of yellow or green discoloration
(3) Positive +	Grey-purple with one to several yellow balls
(4) Positive + +	Yellow-purple often with several yellow balls and adherent sediment
(5) Positive + + +	Green-yellow with considerable adherent yellow sediment
(6) Positive + + + +	Yellow, coagulated, and possibly digested.
(7) Contaminated	Grey coagulated mass often partly digested. May be fluid and olive green without sediment.

The last group—contaminated—can be reduced by the addition of sodium azide to the sample tubes as suggested by Packer (42), but this has been criticized by McLeod & Watts (43), who brought evidence to show that certain strains of *Str. agalactiae* were particularly susceptible to this inhibitor. That streptococci present in milk samples are not always able to grow, even in ordinary laboratory media without inhibitor, is well known, and Cottoni, Forgeot & Thieulin (44) examined such samples for the presence of bacteriophage. None was found and they suggest that serum agar is the best medium for the isolation of the organism from milk. This may be true in fully equipped laboratories dealing with relatively few samples, but in small laboratories or with private practitioners the incubation of the milk samples followed by microscopical examination (the incubated smear method) is probably the most efficient practical method (see Rowlands & Field (45)). Field & Smith (46) compared this method with deep plating of 1080 samples. 24·1% were positive for *Str. agalactiae*, and of these 89·4% were positive to the smear and 85% to the cultural method. It would appear from the literature that this, especially when used in conjunction with the Hotis test (when only the positive samples need be examined microscopically), is widely used in the United States as a reliable practical diagnostic method for use on a large scale (Anon. (47)).

If full laboratory facilities are available more accurate diagnosis can be made by using a serological test. The precipitin test is of universal value but the value of the agglutination test is still under review. Thus Stewart (48) in Australia found that all his strains of *Str. agalactiae* were biochemically typical and gave a precipitin reaction with Lancefield Group B serum. Of 526 strains studied antigenically 1·14% failed to agglutinate with his type sera (slide agglutination). He distinguishes five types, of which none was identical with those described in England. Four were, however, closely related to the English types and one of these was identical with an American type. The remaining type was distinct from any known type. Stewart considers that great emphasis should not be placed on the division of *Str. agalactiae* into serological subtypes, since these reactions may not be stable and all types were found to be equally pathogenic. According to another Australian Report (Anon. (49)) *Str. agalactiae* recovered from herds intensively treated for this disease fail to agglutinate with type antisera. Though biochemical tests are not now greatly employed for diagnosis, Cottoni *et al.* (44) have recently compared several reactions. They used the hippurate test, fermentation of aesculin and mannite, growth at 37° C. in Sherman's methylene blue, in 6·5% NaCl, at 45° C., and resistance to 60° C. for 30 min. They conclude that the reaction of *Str. agalactiae* to any given test is variable and depends, at least in part, on the medium used for culture. The most reliable single test was considered to be the hippurate test. Munch-Petersen, Christie, Simmons & Beddom (50) have recently reported an interesting diagnostic reaction for group B streptococci which they state produces a filtrable thermostable agent capable of lysing

sheep or ox red-blood cells in the presence of Staphylococcus B toxin. This can be used as a plate test on primary isolation.

C. *Treatment*

During recent years two drugs—sulphanilamide and its derivatives and penicillin—have been widely used to treat mastitis. Some interest has, however, been maintained in the earlier drugs, and reference may be made to phemarol (a quaternary ammonium compound) reported by Bryan, Hedeman, Visger & Cunkelman (51) to have cured 147 out of 171 cows; clavacin (derived from *Aspergillus clavatus*), which was considered very unsuitable by Petersen & Graham (52) as it is very irritant to the udder and failed to cure any of nineteen quarters injected, and tyrothricin. The literature on this latter has been reviewed by Little (53), who reports that out of a total of 583 quarters treated by numerous workers 83% were cured. Bryan (54) has claimed that tyrothricin will cure 90% of cases of mastitis if adequate (125 mg.) doses are given and the udder is not fibrosed. Bean, Miller & Heishman (55) also emphasize the importance of the degree of induration. They attempted to eradicate mastitis from six herds by sanitary measures combined with intensive therapy. Treatment was by acriflavine, entozon, colloidal silver oxide and tyrothricin. Of the infected animals 68% were cured while lactating and in the dry cows 78% of infected quarters were cured. In their hands, however, tyrothricin cured only 51% of the quarters, while acriflavine cured 89%. McAuliffe (56) also used acriflavine and tyrothricin for cases of streptococcal mastitis in lactating cows. Silver oxide was used in dry cows. Greene (57) found acriflavine the most useful in a series of 1142 quarters treated. He used tyrothricin mainly in dry quarters. Most authors consider tyrothricin to be non-irritant, but Andberg & Weirether (58) report that it caused an acute mastitis in all animals treated by them. In animals receiving four daily injections this was so severe that milk production had not returned to normal 126 days after treatment.

Since Stableforth, Hignett, Watts, Paterson, Cunningham, Edwards, Malcolm & Roach (59) reported their work on sulphanilamide, in which they showed that, using an initial dose *per os* of 1 g./5 lb. body weight followed by 1 g./30 lb. every 8 hr. for 5–7 days, only 35% of infected cows were cured, interest in the oral administration of this drug has not been great in this country, though it has been widely used for the treatment of clinical cases at a dose of 3 oz./800 cwt. followed by 1 oz./8 cwt. every 8 hr., or 2 oz. every 12 hr. often combined with udder infusion by acriflavine or a soluble sulphanilamide (see Gould (60)).

Sulphanilamide has also been used as an udder irrigant, using different vehicles for suspension. Knodt & Petersen (61) used 600 ml. of freshly prepared 10% suspensions in distilled water, Kakavas, Palmer, Hay & Biddle (62) 40 ml. of a 38% suspension in mineral oil which gave a concentration of 500 mg.% in the milk at 2½ hr. falling to 25 mg.% after 24 hr. This is reported by them to have cured 251 out of 265 cows (94%), a figure comparable to that given by Sanders (63) (90%), using sulphanilamide in iodized mineral oil. Roberts (64) also considers a 30% suspension of sulphanilamide in oil the most satisfactory drug for intramammary injection. Another oil-water suspension, in which lanette wax and arachis oil were used, was described by Edwards & Brownlee (65). They claim a curative rate of 86% in cows treated with 30% sulphanilamide suspended in this mixture and injected daily for 4 days. In an attempt to eradicate mastitis, Edwards, Hughes & Taylor (66) applied this method to all infected animals in a herd of fifty-five cows, of which twenty-four were originally infected. Though the incidence was greatly reduced, they failed to obtain complete eradication. They consider this failure to be due to the persistence of infection on the teat surface and to the retention in the herd of cows resisting treatment.

Sulphapyridine in doses *per os* of between 5 and 7 g./100 lb. body weight three times daily has also been investigated. The blood concentration at these doses is between 8 and 18 mg./100 ml. (Live, Stubbs & Gardner (67)). Toxic symptoms tended to appear early in treatment and the results were not comparable to those of sulphanilamide itself. They report that none of the six cows they treated was cured, while Jaquette, Kleckner & Klein (68) found only ten out of thirty-four quarters cleared.

Francis & Peters (69) studied the efficacy of a wide range of sulphonamide preparations:

sulphanilamide, sulphapyridine, sulphadiazine, sulphamezathine, sulphathiazole, sulphone, sulphonilamide E.O.S., sulphanilamide L.S.F., soluseptasine and solupyridine on *Str. agalactiae*. To do this they first of all elaborated a technique whereby the organism was propagated in chick embryos. It proved pathogenic to these and multiplied rapidly, killing the embryo in about 24 hr. Post-mortem changes were most apparent in the liver and kidneys, the streptococci being mainly observed lining the endothelium of the blood vessels. After serial passage the strain was used to infect mice and by means of serial passage in this animal, using ground liver as the source of the infecting organism, the virulence to mice was raised to 0·1 ml. of a liver broth culture (this was better than blood broth). The sulphanilamide preparations were then tested for their efficacy in controlling the artificial disease (Francis, Peters & Davies (70)). The embryos were infected with a dose of 0·2 ml. of a 50% dilution of a 24 hr. old broth culture of *Str. agalactiae*, and 1 hr. later the sulphonamide compound to be tested was inoculated into the allantoic cavity suspended or dissolved in 0·5 ml. distilled water. The mice were first given the particular drug by mouth and 1 hr. later were injected intraperitoneally with 0·5 ml. of a 24 hr. blood broth culture of the mouse-passaged strain. The doses of the sulphonamides in the embryos varied from 1·25 to 20 mg. and it was found that in the middle of this range the mean specific deaths were related to the log of the dose. In mice the doses were 0·25–2 mg. and again the response curve was linear with the log of the dose.

They found that the complex soluble sulphonamides had the activity theoretically expected according to their content of actual sulphanilamide, and that dose for dose sulphone was the only drug which showed any greater potency than sulphanilamide itself. Schuhardt, Carroll, Rode & Lacy (71) studied eight sulphonamide compounds, of which they found that only sulphanilamide and sulphapyridine were excreted in the milk after administration *per os*. The general therapeutic value of this group of drugs has recently been reviewed by Stableforth (72) and the pharmacology discussed by Hawking (73).

There is a certain proportion (said by Palmer (74) to be 10%) of *Str. agalactiae* species which is resistant to the action of sulphanilamide, but this has not been reported in the case of the antibiotic penicillin. Foley, Lee & Epstein (75), using nine strains, and Watts & McLeod (76), using fifty-six strains, report that this material is inhibitory to *Str. agalactiae in vitro* at approximately the same level (0·02 unit/ml.) as it is against *Staphylococcus aureus*. Packer (77), however, tested thirty-three strains of *Str. agalactiae* and 137 *Staph. aureus*, and found that the majority grew in from 0·03 to 0·06 unit per ml. Heishman (78) gives figures similar to those of Packer, but in his case the basal medium was blood agar which is much richer than the medium usually used. According to Seeley, Anderson & Plastridge (79), the concentration necessary to kill the bacteria *in vitro* varies from 0·3 to 0·18 unit per ml. (only two strains were tested). Concentrations of this order are not obtained in the milk of cows following intramuscular injection of penicillin in economic therapeutic doses, since Seeley, Anderson, Plastridge & Pearson (80), Watts & McLeod (76) and Slavin (81) all record no observable concentration in the milk following intramuscular injections of up to 1 mega unit. Considerable concentrations in the milk have, however, been recorded by numerous workers following intramammary infusion. The actual values given by the various authors have varied greatly, owing partly to variations in technique (e.g. use of whole milk or only fore milk) or to the use of only one or two experimental animals (Weirether, Jasper & Petersen (82), Murnane (83), Bryan, Horwood & Huffman (84), Schalm & Casselberry (85), Stevenson (86), Little & Plastridge (9), Thorp, Uhrik & Straley (87), Kelly & Bell (88), Watts & McLeod (89), and Packer (90)).

The largest group of figures is that reported by Watts & McLeod (89), in which a standard dose was used in 130 animals and the results analysed statistically. The dose chosen was 20,000 units of sodium penicillin in 50 ml. water and they report an average concentration in the bulk milk 12 hr. after injection of 2·6 units per ml. Within the limits of the experimental error of the methods employed the concentration was not affected by the volume of milk, the age of the cow, the stage of lactation or the chloride content. That it was not affected by the volume of milk agrees with Packer (90) but not with Murnane (83) or Schalm & Casselberry (85). Significant differences were found, however, between infected and non-infected cows, the average levels 12 hr. after injection

being 3·2 and 2·2 units per ml. respectively. Figures on a comparable scale for penicillin dissolved in other substances are not available, but a few by Barker (91) and Spencer, Kraft & Underbjerg (92) show no significant differences from those recorded for penicillin in water.

Watts & McLeod (89) also report that in a small series of cows (23) higher levels were obtained by using the penicillin dissolved in only 1 ml. of water instead of the 50 ml. standard dose. (Charts

Table 1. *Penicillin treatment*

(Correlated results of many authors arranged according to the time between injections)

Time interval	No. of injections	Doses in thousands of units	Treated (quarters)	Cured	Percentage cured
	1	<99	402	269	67
		31–99	651	270	41
		15–30	189	92	49
		1–10	93	37	40
12 hr.	2	100	12	11	92*
	4	31–99	30	26	86
		10	39	37	95
	5	15–30	151	116	77
		10	50	34	68
	6	31–99	10	10	100*
		15–30	2	1	—
24 hr.	2	100	26	18	69
		31–99	109	78	72
		15–30	243	180	74
	3	100	18	15	83
		31–99	53	41	77
		15–30	287	214	75
		12·5	28	20	71
	4	31–99	77	66	85
		15–30	97	83	86
		10	5	2	40*
	5	31–99	8	8	100†
		15–30	5	5	100*
4 days	2	100	13	5	38
		31–99	58	27	46
		15–30	18	5	36
		10	2	2	—
	3	100	8	3	37*
		31–99	31	9	35
		15–30	13	8	62
7 days‡	2	100	11	2	18*
		31–99	18	5	36
		15–30	29	17	59
		1–10	53	5	10
	3	100	9	1	11*
		31–99	13	2	18
		15–30	22	5	44
		1–10	48	5	10
12 days‡	2	25	17	7	41
	3	25	10	3	30

* Based on very limited numbers of observations.

† Probably duplicated set of 4 reported in different articles.

‡ These figures include retreatment figures for quarters not cured by previous injections.

given for four cows by Thorp, Uhrik & Straley (87) also show this factor, though the authors do not refer to it.) The lowest levels of penicillin recovery obtained by Watts & McLeod were recorded in experiments in which there was an abrupt decrease in the milk yield following injection. No explanation was found for this, but it could not be related to the batch of penicillin, the diluent or the temperature of the injection.

These workers used two methods of assay in duplicate. The cup method of Florey, Abraham,

Chain, Fletcher, Gardner, Heatley & Jennings (93) and a dilution method using litmus milk. Other workers have used one or other of these two main methods and many reports on the details of the assay of penicillin have been published. It is understood by the present writer that the Oxford group of workers referred to above (93) are shortly publishing a complete review of the subject and so no further reference will be made here.

Penicillin therapy has been widely used in practice and numerous authors have reported on its efficacy. Table 1, slightly amended from Watts (94), gives a summary of the results reported by Kakavas (95), Murphy & Pfau (96), Slanetz & Allen (97), Barker (91), Porter, Campbell, Weber & Reutner (98), Murnane (99, 100), Hardenbrook, Alberts & Bryan (101), Schalm & Casselberry (85), Byrne, Pullin & Konsk (102), Schofield (103), Little & Plastridge (9), Porter & Kelman (104), Schofield & Barnum (105), Watts (94) and Spencer *et al.* (92).

While precautions have been taken to omit duplicated results from this table, it is possible that some have been included, but if so they form but a small proportion of the whole and are mainly in the smaller groups. From all these results it seems that the minimal effective dose is approximately 20,000 Oxford units and that dosages greatly in excess of this do not yield a correspondingly higher percentage of cures. In this connexion Packer's (106) results are interesting. He found that in his cases he recovered the same level of penicillin (using fore-milk samples) 12 and 24 hr. after injection, whether the dose injected was 100,000, 50,000 or 25,000 units. He suggests that there is a 'threshold value' for penicillin and that whatever the dose the concentration does not rise greatly above this value. Daily milk yields recorded by several of these authors show that in therapeutic doses and small volumes of diluent, penicillin is non-irritant in the udder. Schalm & Casselberry (85) and Watts (94) state that the percentage of cures is greater in cows giving small yields of milk and the latter author also claims that the prognosis is affected by the conditions of management of the farm, the age of the cow, the clinical condition of the udder, the number of quarters affected in the cow (see also Porter & Kelman (104)) and the stage of lactation. That this last factor affects the result is denied by Porter *et al.* (98) and by Hardenbrook *et al.* (101), but Casselberry (107) considers that double the dose is necessary if the milk yield exceeds 40 lb. per day. Schofield (108) claims that *Str. agalactiae* is resistant to the effects of penicillin if the gland is considerably indurated and therefore the prognosis is affected by the clinical condition of the gland. Hardenbrook *et al.* (101) and Spencer *et al.* (92) do not agree, holding that indurated udders are as readily cured as relatively normal ones.

There has been a recent tendency to employ penicillin in minimal quantities of diluent. Thus Barnum (109) reports favourably on the results of inserting bougies containing 25,000 units of penicillin, and Watts (94) successfully used 1 ml. of distilled water containing 20,000 units.

A number of reports on the efficacy of penicillin have been omitted from the above account, since for various reasons they are difficult to compare. Among these are: Report Agricultural Research Council Great Britain (110), Report No. 5 Committee of Animal Health U.S.A. (47), Moon (111), Kästli (112), Murphy (113), Downham & Christie (114) and Johnson & Fincher (115).

The whole subject of the treatment of contagious mastitis by intramammary infusions of drugs is discussed in Report No. 5 of the Committee of Animal Health U.S.A. (47) and by Little & Plastridge (9).

D. *Other types of mastitis*

Of other bacterial infections of the udder staphylococcal infections have received the most attention. This may be due to the reports that this disease is occurring particularly widely in herds where chemotherapy is being used to eradicate streptococcal mastitis (e.g. Murnane (100), Bean *et al.* (55)), or it is possible that with the diminution of clinical streptococcal mastitis following the wide use of sulphanilamide and penicillin it is more readily observed and diagnosed.

The main difficulty with this organism is determining whether staphylococci present in a sample are pathogenic, potentially pathogenic, or merely adventitious saprophytes. Thus Schalm (116) reports that he was able to produce gangrenous mastitis by injecting the teat with staphylococci from apparently normal quarters of other cows. In his experiments he found the lactating udder

more susceptible than the dry and suggests that the response of the udder to infection with staphylococci depends on the vascular condition of the organ. Miller & Heishman (117) also established staphylococcal infections by injecting cultures. Using inocula varying from 700 to 30,000 organisms per quarter, they were able to infect twenty-two out of twenty-five quarters. There were differences in the ease with which infection was established which depended on both the cow and the strain of organism. Some strains required repeated reintroduction before they became established and even then only caused a mild type of mastitis, whereas other more toxic strains became established after only a single injection and caused a more severe form of the disease.

Authors differ as to the best criterion for the determination of the pathogenicity of a strain. Vaccaro, Perez & Sepulveda (118) tested 627 strains from diseased tissues and fifty-five from saprophytic sources for their action on mannite agar, pigment production, haemolytic power and coagulase production. None of the saprophytic types were positive to more than two of these tests, while 93 % of the pathogenic strains were. Sixty-eight of these latter were noted as non-haemolytic, but they were correctly classified by the authors as the other three tests were positive. Boe (119) states that the haemolysin test alone is unreliable. In 509 strains of mixed pathogenic and saprophytic origin he found animal inoculation, pigment production and haemolysin formation to be unreliable criteria for pathogenicity. He considers plasma coagulation to be the most reliable single test followed by the fermentation of mannite. The coagulase test is also advocated by Taylor & McDiarmid (120) and by Field & Smith (121). The former authors use rabbit plasma incorporated in a 30 % agar medium as a direct test for coagulase-positive staphylococci. The suspected milk sample is inoculated straight on to this medium. Field & Smith use the rapid slide method, substituting fibrinogen for plasma (see Berger (122)), and state that in their series all pathogenic staphylococci were coagulase-positive. It appears that their laboratory criterion of pathogenicity was the production of a filtrable haemolysin and this itself is considered by Christie, North & Parkin (123) to be the only reliable laboratory criterion of pathogenicity (1027 strains were studied). They state that, by using sheep blood-agar plates for primary isolation, pathogenicity can be determined immediately. Another rapid test is that reported by Chapman (124), who states that pathogenic staphylococci grow luxuriantly on 7·5 % NaCl agar, while non-pathogenic types grow poorly. Most other organisms are inhibited.

Velilla, Faber & Pelczar (125) compared two media, suggested to them by Chapman, on 270 quarter samples and found one 'Staphylococcus Medium 110' to be considerably the better. This medium consists of lactose, yeast extract, mannite, bacto-tryptose, gelatin, NaCl, K_2HPO_4 and agar, and they report that on it coagulase-positive staphylococci were isolated from thirty-one samples. On a more usual medium only eighteen were recovered. All the coagulase-positive strains showed pigment on the 'Staphylococcus Medium 110'. The authors used the slide-clumping technique, with fresh pooled rabbit plasma as a routine test for the presence of coagulase-positive staphylococci, as they found the correlation between this and the longer, more laborious tube coagulase tests almost complete. Beamer, Goodof & Smith (126), after examining fifty-five strains, conclude, however, that none of the usual laboratory criteria are accurate, owing to the variations in the occurrence of positive reactions, and they consider that the clinical findings are the important criteria.

Boe (119) divided his strains into two groups, A and B, on the basis of the precipitin test. Group A included the pathogenic strains and group B the saprophytic. There appears to be disagreement, however, between these groupings and other reactions, and agglutination and agglutinin absorption tests were unreliable, owing to the difficulty of obtaining stable antigens. There were numerous and varied transitional forms. The simplest medium suggested for differentiating staphylococci is the ordinary Hotis test in milk advocated by Schalm (127). This author analysed the results of 2889 samples, comparing the Hotis test, the microscopical examination of incubated samples and direct plating. He found that toxicogenic and coagulase-positive staphylococci were present in 590 samples. If the Hotis test was read at 20 hr., then adherent green, greenish brown, brown or russet coloured colonies with white centres were diagnostic of pathogenic staphylococci. Using this criterion, 58·3 % of the samples containing these were diagnosed, while 8·9 % of false positive

readings were obtained. These readings were combined with the reading at 40 hr. in a series of 1733 samples containing 325 cases of pathogenic staphylococci. A characteristic digestion (shown in colour photographs) occurred in most cases where pathogenic staphylococci were present, and the two readings (at 20 and 40 hr.) taken together detected 80% of the positive samples, with 7·1% false positive results.

Complete differentiation between staphylococci of bovine origin is likely to be difficult, as MacDonald (128) found that all the staphylococci in over half of 280 samples of milk were of one provisional phage type (42 D). If they are as closely related as this evidence suggests, biochemical tests cannot be expected to reveal any definite distinctions between strains. Mastitis due to staphylococci is more difficult to treat than streptococcal mastitis, since staphylococci are resistant to the acridine dyes and sulphanilamide. Treatment with intramammary injections of sulphanilamide in oil has been advocated by McAuliffe (56), but most authors do not agree. Roberts (64) suggests drying off any affected quarters after the acute stage is past by injecting 50–100 ml. of a 1–3% solution of silver nitrate which is left in for 2–3 weeks. Tyrothricin also is of little value, while reports on the efficacy of penicillin have varied. Klein, Crisman & Moor (129) succeeded only after eight successive injections of 22,800 units, and Little & Plastridge (9) record only twenty-four quarters cured of ninety-seven treated (varying doses), and again their percentage of success was greatest where multiple injections were used (four or five), though Murnane (100) records no success (only two cured out of twenty) using six injections of 25,000 units. McCulloch (130) attempted to treat ten quarters with chronic staphylococcal mastitis by four intramammary infusions of 100,000 units at intervals of 12 hr. A temporary reduction occurred in the number of staphylococci shed but the disease was not cured. On the other hand, Slanetz & Allen (97) claim to have cured four cows using four injections of 100,000, and Walker (131), using 50,000 units in a 3% beeswax-peanut oil base, cured six quarters with six successive injections at 12 hr. intervals. Field trials were variable and no summarized figures are given, but the author concludes that if large doses are given and repeated a considerable degree of success is obtained. That large repeated doses, while valuable, are not always successful, is shown by Murnane's (100) results where staphylococci highly resistant to penicillin were found. This author also found this disease appearing in herds where mastitis caused by *Str. agalactiae* was greatly diminished by treatment, and this has also been the present writer's experience. In herds where *Str. agalactiae* has been eradicated by using repeated (5) doses of 100,000 units, staphylococcal mastitis has been repeatedly diagnosed, often causing clinical disturbance, many of the strains being relatively resistant to penicillin. This shows that even so large a dose as 100,000 units is not sufficient to eliminate the staphylococci from the udder or that, if they are eliminated, they rapidly reinfect the udder.

On theoretical grounds staphylococcal mastitis should be more difficult to treat than streptococcal, since staphylococci resistant to penicillin, i.e. capable of producing penicillinase, are readily produced in human staphylococcal infections (for references see Murnane (100)). Murnane (100) states that penicillinase production was the factor responsible for the resistance developed by his strains. Packer (77) was unable to show any increased resistance in strains of staphylococci recovered from treated cows, but reports that from 7 to 50 Oxford units/ml. were necessary *in vitro* to kill *Staph. aureus*. These figures are similar to those of Seeley *et al.* (79).

Demerec (132) states that resistance to penicillin in staphylococci isolated from treated cases is not induced by the actual action of penicillin on the organisms, but is a definite mutation. In any given population there is a proportion resistant and this proportion survives and mutates. Foster & Wilker (133) suggest, however, that inhibition *in vitro* is due to long prolongation of the time of generation, i.e. to slow multiplication of all the organisms and not to the more rapid multiplication of a small resistant group.

Staphylococcal toxoid has been used in the treatment of this disease and is of some value. Its use is advocated by Gould (60) and Murphy (113), though neither give definite figures. Miller & Heishman (134) are, however, doubtful of its value, mainly on theoretical grounds. They examined 220 cows and found no antitoxin in the blood of fifty-seven (25·9%), up to 4 units in 110 (67·4%),

and 5–64 units in fifty-three. No true correlation was found between the antitoxin titre and current staphylococcal infection, but clinical cases gave the highest titres. Antitoxin was found in only ten out of sixty samples of milk. In each case there was less than 1 unit and the blood titre was from 16 to 96 units. Two clinically affected quarters (acute mastitis) showed 16 and 32 units respectively, though the healthy quarters of the same cows showed only 1 unit or less. Where antitoxin was present in the blood, the colostrum gave a high unitage, though this fell rapidly after parturition. Since quarters which have shown high titres of antitoxin during the dry and colostral periods still harbour staphylococci, the authors conclude that theoretically staphylococcal toxoid is unlikely to be of therapeutic value.

The treatment of mastitis due to the *coli-aerogenes* group of bacteria has been described by Burkhardt *et al.* (24), Knodt & Petersen (61) and Weirether, Jasper & Petersen (135). As would be expected, penicillin was found useless against this disease (135), but Knodt & Petersen (61) report that 600 ml. of a 10% suspension of sulphanilamide in water cured three acute and two chronic cases of coliform mastitis. In the outbreak treated by Burkhardt *et al.* (24) *Bact. aerogenes* introduced into the herd by a carrier cow was incriminated. Of the thirty-three cows in the herd, eleven showed acute toxaemic mastitis, which if treated early with sulphanilamide responded within 4 days, but if treatment was delayed for 12–18 hr. after symptoms had appeared cure was delayed for 10 days.

Attempts have been made to treat *Corynebacterium pyogenes* mastitis with penicillin. Although the organism is susceptible *in vitro* (Downham, Christie & Hughes (136)), no success has been recorded in actual treatment even with very large doses. These authors and Arthur (137) used 1,000,000 units, repeated three to eight times, without influencing the course of the disease.

Murphy & Drake (138) report an interesting outbreak of mastitis due to a new species of yeast. Over a period of 6 years ten cases were observed, mostly of a chronic clinical type, while Packer & Merchant (139) have encountered nine cases of mastitis due to *Pasteurella* organisms typical of *Past. muricida* group 2. An unidentified *Corynebacterium* resembling *Corynebact. bovis* was found by Palmer, Kakavas & Biddle (140) in twenty cows in a herd of sixty. The clinical symptoms were swelling of the quarter with occasional oedema followed by partial induration. Mastitis due to a Lancefield group A streptococcus, which caused an epidemic of human sore throat, has been reported by Andberg & Weirether (141), while Pfeiffer & Viljoen (142) record a similar epidemic of diphtheria due to *Corynebact. diphtheriae* in two cows.

II. CONTAGIOUS ABORTION

A. *Bacteriology*

The study of the bacteriology of *Br. abortus* has been concentrated mainly on Strain 19. This will be referred to in more detail under Vaccination, but some general points will be included here. It has long been known that the *Brucella* group is readily divisible by its resistance to various dyes. McLeod (143) found that this could be used to differentiate between strains of *Br. abortus* itself. Using thionine blue as the inhibitor, he reports that 1 mg. per litre of bactotryptose agar completely inhibits Strain 19 but allows other strains to grow. He emphasizes that different batches of dye vary but that every batch is similar in its effects, even though the threshold value varies. Small inocula should be used. His work has been confirmed by Levine & Wilson (144). Gerhardt & Wilson (145) found that, while *Br. abortus* Strain 19 would not grow in ordinary synthetic media using $(NH_4)_2SO_4$ as the source of N_2, it was able to grow if lactate was used as the energy source instead of glucose or glycerol. Relatively heavy inocula were required to initiate growth, but the authors did not consider that the inevitable organic carry-over affected the results, as serial passage soon diluted this out.

According to Moore & Mitchell (146) *Brucella* can be grown satisfactorily on a 'solid' medium not containing agar. This consists of dishes in which cotton-wool is packed and impregnated with glucose broth + 10% brewers' yeast hydrolysate. On top of the cotton-wool a layer of dialysing paper is placed, wetted with the medium, and the whole sterilized. The paper is then seeded with

5 ml. of a liquid culture and incubated. Satisfactory yields of *Brucella* suitable for use as antigen were recorded.

As Huddleson (147) points out, *Br. abortus* is an organism which dissociates readily, though often this dissociation is only revealed by examination of the colonies on a transparent medium using a stereoscopic microscope with transmitted light. He distinguished three different mucoid phases, all of which were antigenic and produced immunity in experimental animals. This phenomenon has been closely studied by Braun (148) employing strain 19. By using single cell isolates he was able to demonstrate clearly that the percentage dissociation was much closer about the mean in closely related colonies, i.e. daughter colonies of a single cell, than in colonies derived from the same culture but not the same cell. From this he argued that the degree of dissociation is genetically controlled but is subject to environmental influences particularly those which affect the growth rate and viability (e.g. pH of medium, temperature variations, available nutrients, O/R potential and repeated sub-culture).

Braun & Bonestell (149) were able to distinguish rapidly between the various phases of *Brucella* by using acriflavine. 0·1 ml. of a 1:1000 aqueous solution of acriflavine was added to 1 ml. of a saline suspension washed off agar. With smooth cultures the tube remained cloudy, but if rough a flocculent precipitate appeared which settled within 1–2 hr., leaving the supernatant liquid clear. Mucoid variants produced slimy precipitates, and intermediate types varied from a precipitate to a granular deposit. They used this technique to furnish evidence for their thesis that dissociation is due to the spontaneous undirected appearance of variants (mutation) and their subsequent establishment under selective pressure. According to Sanders & Rice (150), dissociation can be prevented by growth in a basal medium of tryptose, glucose and thiamine hydrochloride, agitated during growth by air, oxygen and carbon dioxide. Aeration prevents dissociation, but if oxygen was used no growth occurred.

Possibly associated with dissociation is the phenomenon reported by Polding (151) of germination 'breaks' in *Brucella* cultures. Using liver agar he found that while bacterial dilutions of 10^{-1} or 10^{-2} would germinate totally, dilutions of 10^{-3} failed to germinate at all. The 'break' was abrupt however close the dilutions and did not occur in all batches of liver agar or in tryptose agar. He studied the phenomenon by modifying (*a*) the technique of preparation of the liver agar; (*b*) the method of diluting; (*c*) the culture media; and (*d*) the pH. He also investigated the effect of cell metabolites, using for this purpose disks of liver agar superimposed on plates of this medium. In one series the plates were poured with 'defective' agar inoculated with serial dilutions of *Br. abortus*. When the agar had set, a disk of heavily seeded liver agar was placed centrally on each dish and incubated. Growth in the 'defective' medium occurred only round the disk. In a second series he reversed the media, using non-defective medium on which he superimposed disks of 'defective' medium. In this series no growth occurred round the disks. In each case the diameter of the zones round the disk depended on the number of bacteria present in the main medium.

He concludes that during the preparation of liver media a bactericidal inhibitor is produced to which various strains of *Br. abortus* respond variously. Live germinating cells produced an 'anti-inhibitor' which could also be demonstrated in two fractions of blood. This anti-inhibitor was not a vitamin and the author suggests that actively growing cells synthesize this factor and that it is connected with virulence. A substance known to inhibit *Br. abortus* and to alter its characteristics is para-aminobenzoic acid. Cotton & Swope (152) found that 2 mg. per ml. completely inhibited the growth on tryptose agar while 3 mg. per ml. sterilized a suspension of 46×10^6 per ml. in distilled water in 24 hr. In broth, 5 mg. per ml. did not kill all the organisms within 35 days, but higher concentrations killed in from 3 to 6 days. In broth the sodium salt of P.A.B.A. was more effective than the parent substance, but in water the reverse was the case. The authors (153) then examined the therapeutic value of the drug in guinea-pigs, using a dose of 5 ml. of 1% NaP.A.B. subcutaneously every 4 hours. The animals were divided into groups of 5, some treated for 72 hr. before infection, some not treated till 72 hr. after infection and some not treated till 14 days after infection. The infecting dose was $1{\cdot}5 \times 10^9$ virulent *Br. abortus* injected intraperitoneally. All

animals were killed 38 days after infection and examined for gross lesions and for the presence of *Br. abortus* in the organs. The results varied with the method of treatment but gross lesions were entirely absent in the treated guinea-pigs, and while *Br. abortus* was occasionally isolated in culture, only two to six colonies were obtained per plate as compared with the heavy growth from control animals.

De Ropp(154) has studied the effect of infecting 9-day-old chick embryos with strains both virulent and avirulent to the guinea-pig. He used a fully virulent smooth strain, an avirulent smooth strain and three avirulent rough strains. Both the smooth strains were fully virulent for the embryo, while none of the rough strains were infective even in doses of 6×10^8. The virulence for a strain for cattle is also not paralleled by its virulence for mice. He has also confirmed the previous work on the infectivity of *Br. abortus* for this animal and has shown(155) that, while a fully virulent (cattle) strain propagated slightly more readily than a less virulent strain, this distinction was much less noticeable than in guinea-pigs. Using a dose of 300,000 organisms, no mice were killed but persistent infection was readily set up, especially if the animals were inoculated by the intracerebral or intravenous routes. Intramuscular or intraperitoneal injections were not so consistent and if only 300 organisms were injected only a slight non-progressive infection resulted. Agglutinins could be demonstrated 5 weeks after inoculation with the larger dose. Using much larger doses of bacteria (3 to 5×10^9) Gonzalez(156) reports that repeated intraperitoneal passage in mice enhances the virulence till this dose will kill within 24–96 hr., though this increase in virulence is more readily obtained using *Br. melitensis* than with *Br. abortus*.

Karsten(157) also used this level of dosage to determine virulence. He considers that this dose of a fully virulent strain recently isolated will kill mice (intraperitoneal injection) and that if the mice survive, the degree of virulence can be estimated by the lesions in the organs. The virulence so judged diminished on prolonged culture in artificial media. Whether mice are satisfactory laboratory animals for work on *Br. abortus* remains doubtful, since in all these reports the infective dose is huge compared with that used in guinea-pigs, e.g. Hutchings & Huddleson(158) used only 10 to 100 organisms subcutaneously to produce macroscopic lesions in the liver and spleen and a blood agglutination titre within 30 days. These same authors found that 5×10^6 organisms instilled into the conjunctival sac of pregnant heifers caused infection and abortion. While infection does not always cause abortion, it certainly affects the reproductive life of the cow. Live & Stubbs(159) studied this effect in 106 cows. They divided them into four groups: (*a*) sixty-three cows positive to the agglutination test; (*b*) twenty-three cows all negative to the blood test when added to the herd; (*c*) these twenty-one cows after their addition to the herd; and (*d*) twenty-two heifers similarly negative before addition. They compared the breeding life of the different groups from the time of their first calf till slaughter. Group (*b*) was the control group and showed a time interval between calvings of 11 months. All the other groups averaged 15–16 months with an incidence of retained placenta of two to three times that of the control group, though actual abortions never exceeded 60% in any of the groups.

Infection can be spread by artificial insemination from infected bulls, since Seit(160) has recorded forty-nine cows infected out of 921 inseminated from one bull. All these forty-nine were inseminated on two successive days. The bull was slaughtered 4 months after seminal tests had revealed active brucellosis, but no abnormality of the organs of the genital tract was found except for signs of a previous inflammation of the ampulla of the left deferent duct (Bendixen(161)). Thus the bull had recovered rapidly and had only been infective for a very short period.

Bendixen & Blom(162) examined 1109 specimens of semen and 552 blood samples from 394 bulls. Fifty-eight of these bulls gave a positive blood reaction and thirty a positive semen agglutination. This latter test indicates a focal lesion in the genital organs and in fifteen of them *Br. abortus* was demonstrated in the semen.

Further evidence of the localization of *Br. abortus* in the udder of milking cows has been published by Jones(163) who found 15% of 722 bulk milk samples to be infected with this organism. He states that the incidence was highest in January and lowest in August.

Milk is not the only source of human infection from brucellosis. In Chicago, Levine(164) reports

that of twenty-four cases of acute brucellosis seventeen were patients employed in meat-packing stations. After considerable questioning they admitted they habitually ate portions of half-cooked meat or meat products, and Levine considers that in this type of case, infection is by actual ingestion of infected material and not by external contact only. Purriel, Risso & Espansandin (165) report similar infection in workers in meat-refrigerating plants. In six cases acute epididymitis occurred with subacute prostatitis and vesiculitis in some cases. (It is not clear from the reports available whether these were infections of *Br. abortus bovis*, or *suis* but quite possibly they were the latter.) Staub (166) estimates that 10–15% of the American population are infected with brucellosis, chiefly those employed in the dairy and livestock industry and its auxiliaries. Jordan (167) gives figures for each group of workers in Iowa estimated as annual rates of *Brucella* infection per 100,000. From the paper it appears that all types of brucellosis are included (i.e. *bovis*, *suis* and *melitensis*). His figures are: Children under 12—rural 0·9, urban 0·4. Teen ages—rural 0·9, urban 0·3. Adult females—rural 2·2, urban 1·4. Adult males—farm worker 43·0, merchant-professional (urban) 3·3. Packing-house workers 271·5. Veterinary surgeons 250·0. He states that the disease is more prevalent from April to September than from October to March. In England Soltys (168) tested 1392 human sera (received for Wasserman tests) and records 3·7% yielding a higher titre to *Brucella* than 1:20, while a few had much higher titres suggestive of active infection. *Brucella* antibodies are also present in the colostrum of actively infected cows and these are passed to the calf on ingestion of colostrum. According to McDiarmid (169) they can be demonstrated in the calf's blood in from 1 to 3 hr. following ingestion, reaching a maximum at 24 hr. They then decline as a logarithmic curve, the period of persistence depending on the original titre of the colostrum.

B. *Diagnosis*

Diagnosis is still mainly confined to the blood agglutination test. Olsen (170), however, found that twenty-four out of 743 (3%) of placentae from cows negative to the agglutination test contained *Br. abortus*. The corresponding figures for 334 reacting cows was only 15%, a proportion which would seem to imply that a much greater number than 3% of the non-reacting group were actually infected. Wise & Kerby (171) isolated the organisms from the blood of infected animals by incubating citrated blood with bacto-tryptose broth. After 6–7 days they subcultured from the blood-broth interface and the blood itself, discarding the broth first. Variable results were obtained, the blood on the whole being preferable, though sufficient samples of this material were negative to make it necessary always to culture the blood broth interface as well. The broth itself was never positive unless one of the other two was positive.

Some 15 to 45 days after agglutinins are first demonstrable the complement-fixation test becomes positive, followed after a further 0–30 days by the allergic reaction (Bessenov (172)). This author states that 3–5 years after the original infection 7·7% of animals are positive to the agglutination test, 15·6% to the complement-fixation test and 77·1% to the allergic test. To determine the importance of these reactions he studied three groups of cows:

(1) Reactors to the blood test—thirty-three calvings, eleven abortions, nine foetuses examined, six positive cultures of *Br. abortus*.

(2) Non-reactors to the blood test but reactors to the complement-fixation and allergic tests—thirty-seven calvings, two abortions, both positive for *Br. abortus*.

(3) Reactors to the allergic reaction only—number of calvings not available, no abortions.

Samples of milk from cows in each of the groups showed 24·3% infected in the first group, 1·9% in the second and none in the third. The author concludes that reactors to the complement-fixation test are definitely infective animals and should not be allowed in contact with susceptible animals.

A new test for the presence of active infection has been suggested by Huddleson, Wood & Cressman (173) and Huddleson, Wood, Cressman & Bennitt (174). This test consists of incubating for 24–48 hr. tryptose broth cultures seeded with 150,000 organisms to which plasma from the suspected animal has been added in varying concentrations from 1:10 to 1:1280. Control tubes

without the plasma show turbidity, and the action of the plasma is measured by the degree of turbidity compared with these controls. If the animal is infected, no inhibition of growth occurs, but if it is normal or has been infected and recovered, then the plasma contains a bactericidal complex which kills *Br. abortus*. This complex is also present, though to a lesser degree, in serum, and makes its appearance after the ingestion of colostrum by the calf.

The bactericidal action of serum has also been studied by Irwin & Beach(175). Instead of estimating its effect by turbidity in liquid media they used solid tryptose agar and compared the number of colonies growing on the serum plates with those on control plates. They added complement from known uninfected cattle. They found that Strain 19 was more susceptible to the action of serum than was a fully virulent strain and that vaccination markedly increased the antibacterial action. The serum from normal animals was slightly bactericidal at dilutions of 1:40 to 1:80 while, even after no agglutinins were demonstrable, the serum of vaccinated animals was antibacterial at a dilution of 1:1280. This factor is therefore independent of the agglutinin content of the serum, but according to Richard(176) the antibody content of a serum is closely related to its opsonocytophagic activity. He carried out agglutination and opsonic tests in parallel, and showed that if the agglutination titre was 1:16 or above, phagocytic activity was marked and that as the agglutination titre rose so did the phagocytic activity. Since the agglutination test is much simpler to perform and gives as much information as the opsonic test he considers it the method of choice.

Similar results are reported by Le Grow(177). He divided fifty-four cows and forty-one calves into six groups. Group 1: eight cows with a history of long exposure to brucellosis. Group 2: eleven infected animals. Group 3: eighteen with a low fluctuating agglutination titre. Group 4: seventeen experimental animals previously vaccinated. Group 5: sixteen calves (vaccinated). Group 6: twenty-five calves not fed colostrum before examination. He concludes that: (*a*) the opsonic index is not of itself a criterion of susceptibility or immunity; (*b*) a high index is correlated with a high degree of immunity; (*c*) in most cases a low index together with a low agglutination titre indicates susceptibility: (*d*) vaccination causes a rise in the index coincident with the rise in the agglutination titre; (*e*) the highest degree of immunity occurs just after the agglutination titre has returned to normal and before there is any marked drop in the opsonic index; (*f*) there is no placental transference of opsonins from dam to calf.

In Scandinavia there is much interest in the ring test (milk and stained antigen). The literature on this test has been recently reviewed by Jørgenson(178), who considers that the antibodies demonstrable in the milk come from the blood and are not of local origin. Norell & Olson(179) compared this test with the slow blood-agglutination test in 225 herds. Every case which was positive to the blood test was positive also to the ring test, but there were eight positive ring tests negative to the blood test. The authors do not regard this as significant, as the two tests were not performed simultaneously. They give the technique in detail and urge its use as a simple routine test in eradication schemes to eliminate negative herds from blood testing. Seit & Jørgenson(180) also found 96% agreement between the ring test and the agglutination test in a series of 3753 samples from 3362 farms. Of these 465 were positive to both tests, fifty-seven were positive to the ring test and negative to the blood test and seventy-two were positive to the blood test and negative to the milk test. It is not clear if these last results were followed up to ascertain which was correct but as they were carried out in successive years and not simultaneously it is unlikely that this point could be determined.

Live and his co-workers (Live, Stubbs & Gardiner(181), Live, Sperling & Stubbs(182) and Stubbs(183)) have reported an allergic test using an injection of 'sonic filtrate'. This is produced by exposing a culture of *Brucella* to 9000 vibrations per sec. to kill the organism and then filtering. As a test for specificity, 0·1 ml. of filtrate (0·125–0·5 mg. protein) was injected into the caudal fold of seventeen cows assumed to be free of infection. No reaction was observed. To test the reaction in infected cows, sixty-three old reactors, forty-one of which were still positive, and fifty-four non-reactors added to the reacting group, were further blood and skin tested. The various groups reacted as follows: (1) Old reactors still positive to blood test, 41; positive to skin test, 33. (2) Old

reactors doubtful to blood test at time of experiment, 17; positive to skin test, 17. (3) Old reactors negative to blood test, 5; positive to skin test, 3. (4) Old negative or doubtful blood reactions positive to blood test, 35; positive to skin test, 33; doubtful or negative to blood test, 14; all negative to skin test. Their conclusions are that the skin test does not yet fulfil all the conditions for a field test, especially since it does not always agree with definite blood reactions. The injection actually produces agglutinins itself, though these disappear in about 7 weeks. A non-agglutinogenic filtrate has been described by Live & Stubbs(184), but it only detected 54·7% of 190 cattle positive to the agglutination test in herds with long-standing brucellosis and gave positive results in 24·8% of 177 cows negative to the blood test.

C. *Control*

While in Britain no effort is made to eliminate brucellosis by the elimination of infected stock, such schemes are in progress in Scandinavia and in America. Thomsen(185) reports in detail on the Danish scheme. By October 1944, 340 out of 1661 dairying districts had been tested, comprising 42,384 herds containing 441,284 cattle. 10,618 (25%) of the herds contained reactors and 11% of the cattle reacted. Petersen & Seit(186) state that the scheme is based on (1) blood testing all stock, (2) conservation of clear herds, (3) removal of reactors in herds with only very few reactors, and (4) segregation and vaccination of all herds with a high proportion of reactors. They found the prompt removal (sale) of reactors the most effective way of freeing a herd, since isolation was rarely successful. Some herds cleared themselves, however, even without much effort or care on the owner's part. 3790 herds were enrolled in their scheme. They were mainly small herds with less than ten milking cows. The initial test showed 1259 (33·2%) of the herds infected. During a 5-year period, 461 previously free herds became infected and 1046 were cleared. At the end of the period 674 were infected. The incidence had therefore been reduced by about 50% during the period (from 1259 infected farms to 674).

Seeleman(187) also considers the elimination of high titre animals necessary even if vaccination is employed, while Wille(188) does not think vaccination will prevent infection, as he considers that the local immunity of the uterus is important and good feeding and housing are also factors affecting the resistance of the animal to infection.

Crawford(189) states that in America the ultimate aim is to eradicate brucellosis and that the slaughter of infected cattle is the final aim. Meanwhile, in early cases, especially in pedigree stock, vaccination is valuable as it is also in beef cattle under range conditions. In certain cases vaccination of adult cattle is justifiable, even though this may lead to abortion, since such abortions are less serious than natural abortion, Strain 19 disappearing from the genital tract and udder before the next pregnancy. Winter(190) states that in New York State only 300 herds remain on the test and slaughter programme, while 2500 have been transferred to the calfhood vaccination plan with Strain 19. According to an American report (Anon.(191)) there were 2,200,000 herds with 16,800,000 cattle under some type of brucellosis control in the United States in 1943. Of these 846,000 cattle in 40,500 herds were completely free of the disease. Miller, Wight & Crawford(192) have given a review of the position in the U.S.A. in June 1944. They state that in the 12 months to that date 5,235,000 cattle had been blood tested, of which 226,000 were reactors (4·3%). According to Wight(193) this had increased somewhat during the next year to 4·7% (243,050 out of 5,213,458 cattle), but the number of accredited herds and cattle increased by 2645 and 43,188 respectively to 45,314 and 881,496. The majority of reactors were slaughtered under the test and slaughter policy, which is still regarded as the method of choice, but 75,000 in 1944 and 86,738 in 1945 were retained in herds where calfhood vaccination was employed. Simms(194) reports that in 1946, 43,799 herds with 838,395 cattle were free of brucellosis. 4,876,866 tests in 380,814 herds were made and 245,786 (5·0%) of the cattle reacted. 170,864 were slaughtered and 74,922 were in herds where calfhood vaccination was practised.

D. *Vaccination*

The two biggest difficulties in preparing living vaccines against *Br. abortus* are the dissociation of the culture during growth and its instability during storage or transport after preparation. Braun (195) selected clones with a low dissociation index and found that if serum or plasma from normal cows, rabbits, pigs or goats was added to the broth, dissociation was suppressed. Seeleman & Meyer (196) state that if the vaccine is prepared from liquid cultures, the number of viable organisms is less for any given opacity than if the vaccine is prepared from solid media. They consider (197) that glycerine agar is the medium of choice. They used this to prepare four different vaccines which they washed off with (*a*) buffered saline, (*b*) buffered saline + glycerine broth, and (*c*) glycerine broth. The original viable counts varied between 2·4 and $4{\cdot}4 \times 10^7$ per ml. and the authors record that when kept at 12–15° C. the vaccines kept well for about 2 weeks (as judged by viable counts and by virulence tests in mice and guinea-pigs). Some deterioration occurred during the following 2 weeks, becoming marked after the 4th week. Glycerine broth was the best suspending medium. An Australian report (Anon. (198)) also states that the prescribed buffered saline does not prevent a drop in pH and they suggest that in some cases zinc is leached from the rubber stoppers and kills the bacteria. They advocate sterile skimmed milk as the suspending fluid. Verwey (199) also suggests that either this material or serum be used as the suspending fluid in the preparation of lyophilized vaccine. He finds such a vaccine very stable once the initial drop has taken place. This drop reduces the numbers of viable organisms by some 50% in serum or milk but by about 99% in saline. Verwey & Scheidy (200) state that this form of vaccine is approved by the U.S. Bureau of Animal Industry and is available commercially. At refrigerator or room temperature it has an efficient life of at least 3 years, though at higher temperatures, e.g. 100° F. it deteriorates more rapidly, though even at this temperature it is still viable for 6 months. Crawford, Anderson & Love (201) state that the Bureau of Animal Industry has now laid down a 3-month expiry limit on Strain 19 vaccine. The number of doses issued by the Bureau is limited to 200,000 per annum, the balance needed being supplied by commercial houses. Of this balance (3,238,675 doses in 1945) approximately 7% is rejected by the Bureau after standardization tests.

The dosage of Strain 19 vaccine, as laid down by the U.S. Bureau of Animal Industry and followed in Britain and other countries using this vaccine, is 5 ml. of suspension containing $8\text{–}12 \times 10^9$ organism per ml. This dose is considered too large and unwieldy by several workers, and recently Campbell & Rodwell (202) have investigated the value of smaller doses administered intracaudally into the *tip* of the tail and intracutaneously. They injected twenty-six animals with 5 ml. subcutaneously as controls and compared the results of intracaudal injection of 1·0 ml. (thirty-seven animals), 0·2 ml. (twelve) and 0·04 ml. (twelve) with 0·2 ml. intracutaneously (nine), 1 ml. subcutaneously (twenty-six), 0·2 ml. subcutaneously (ten), and 0·4 ml. subcutaneously (nine). They found that the agglutinin response at 14 days was closely related to the dosage and to the route of inoculation. One ml. intracaudally was more efficient than the full 5 ml. dose subcutaneously. It was also more easily performed and did not cause severe or persistent local reactions. In milking cows there was a transient drop in milk yield, but this was certainly not greater than that following the 5 ml. dose. They consider that doses as low as 0·04 ml. intracaudally are as efficient as 5·0 ml. subcutaneously. Following vaccination of adult animals Strain 19 was not found in the milk, and eighty-nine out of ninety pregnant cows calved normally. These smaller doses are not in use on any large field scale, the main results reported so far being with the standard American dose of 5 ml.

Miller *et al.* (192) state that 392,232 calves were injected in America under the official scheme but a large number were also treated unofficially, vaccination being in use even in herds free of abortion, a practice which they themselves advocate. Calves are not blood tested before or after vaccination, which is best performed at 6 months. They consider bull calves should also be vaccinated and all vaccinated calves identified. In addition to calfhood vaccination whole herds are being increasingly vaccinated. In contradistinction to Campbell & Rodwell (202), they report that pregnant animals may abort, and quote a herd of sixty of which twenty-three aborted following vaccination

(Strain 19 was identified as the causal organism). On the other hand, they state that while the vaccine is not 100% effective it has never been shown to increase in virulence nor to infect a free animal from a vaccinated animal. Haring & Traum (203) state that the best results are obtained in herds where heavy losses are occurring before vaccination starts. In these they use adult as well as calfhood vaccination for the first year and then follow with calf vaccination only. They record an abortion rate of 0·9% in 1005 pregnancies of animals vaccinated as calves, though virulent *Brucella* infection has appeared in herds practising calfhood vaccination. In one herd, for example, 13·9% of the animals which were vaccinated as calves, 16·9% of those which were vaccinated as adults, and 40·9% of the unvaccinated animals aborted. Winter (204) reports that in New York State, where careful records have been kept in a group of twenty-five herds for 6 years, there is no record of delayed oestrus or conception or any abortions in any animals vaccinated as calves and whose post-vaccinal blood titre did not return to normal. He vaccinates at 6–8 months and states that 60% are negative at 12 months, 80–90% at 18 months and 96·5% at 24 months. He breeds all his animals, whether they still have a positive titre or not.

Beach, Irwin & Berman (205) tested the resistance of twenty-three animals which were pregnant with their third calf and which had been vaccinated with Strain 19 as calves. They also tested ten non-vaccinated animals. A dose of thirty million virulent *Br. abortus* was used instilled into the conjunctiva. These animals had been maintained since birth in an abortion-free herd and at every calving the membranes, etc. had been examined biologically and culturally for *Brucella*. Blood cultures were regularly made following infection, heparin being used as the anticoagulant instead of citrate, since the latter enhances the bactericidal power of serum. All ten control animals aborted and *Brucella* were isolated from each. In the vaccinated group twelve aborted, two calved early with weak calves and nine produced living calves at full term. They found that, although many of the animals yielded positive blood cultures, this was not sufficiently accurate to be used as a test for infection. The sera were tested for bactericidal activity, but no relation could be found between this and the resistance of the animals to infection when exposed to the virulent strain. They conclude that the protection afforded by calfhood vaccination is less at the third pregnancy than the least that can be expected at the first.

Although calfhood vaccination prevents abortion, especially in early pregnancies, Birch, Gilman & Stone (206) state that it fails to prevent actual subclinical brucellosis in a very large percentage of animals in infected herds. They vaccinated the calves in a large herd at 4–8 months old and kept some of the animals as controls. Monthly blood tests and routine examinations of the milk and uterine contents were made. As the herd was heavily infected, frequent contact with infected stock among the adult stock was ensured. The experiment lasted over six pregnancies. The vaccinated animals showed a much lower abortion rate, a lowered infectivity and a delayed onset of abortion (mainly in the 4th and 5th pregnancies). They showed, however, a very high (53·3%) infection rate, but little below that of the controls (66·77%). It would seem that the resistance acquired following vaccination is relative rather than absolute, but even this is valuable if a 'test and hold in isolation' policy is being pursued. To eliminate the disease using vaccination, all known reactors in the adult stock should be segregated and chronic reactors removed. Trudel (207), who states that vaccination was introduced into Quebec in 1940, agrees closely with Haring & Traum (203) on the rate of abortion following vaccination. In 1500 vaccinated calves followed through to their first or second lactations, the incidence of abortion was 0·86%. He considers that 6–8 months is the best age to vaccinate, since 67·6% of his abortions occurred in animals vaccinated at 4–6 months. Buddle (208) reports a slightly higher figure for New Zealand: of 10,000 heifers vaccinated at over 4 months old, 3% aborted (22% of the unvaccinated heifers aborted). He states that 43,000 calves were vaccinated in 1944 and 107,000 in 1945.

In Britain Strain 19 has been widely used since 1944, when a calfhood vaccination scheme was started by the Ministry of Agriculture and Fisheries. By 1948, 1,126,753 doses had been issued under this scheme and 1,553,627 further doses to private practitioners, a total of 2,680,380 (Lawson (209)). At first this scheme included a choice of vaccine Strain 45/20 McEwan, a rough strain already widely applied under controlled field conditions being also issued. This latter

strain had the great advantage that it did not stimulate agglutinins and that vaccinated animals did not therefore react to the blood test. A series of articles comparing the efficiency of the two vaccines has been published by McEwan, Edwards, de Ropp, McLeod & McDiarmid. De Ropp(210) compared the immunizing powers of the two strains in the guinea-pig. Neither multiplied extensively nor produced observable lesions, though 45/20 persisted longer in the spleen and Strain 19 stimulated agglutinin production. While neither rendered the whole group of guinea-pigs completely resistant to even a very small infecting dose (300 organisms) the amount of infection judged by the estimated viable count of the spleen was considerably less than in unvaccinated controls, especially with Strain 19 vaccine, which also produced immunity more rapidly than did 45/20.

Following this work, the two vaccines were compared in cows by Edwards, McDiarmid, de Ropp & McLeod(211,212). Fifty heifers negative to the blood test were divided into five groups of ten, each housed in a separate bird-proof building with a separate attendant. Two groups were vaccinated with Strain 19 and three were unvaccinated. When 5 months pregnant, the two vaccinated groups and two of the unvaccinated were exposed to infection by conjunctival instillation of 15 million (two groups) and 150 million (two groups) organisms. Blood samples were taken frequently, and at parturition the foetal stomach contents, the colostrum and a cotyledon were examined culturally and biologically. All the control animals at both doses developed infection, producing only four living calves with an average duration of pregnancy of 224 days. Of the eighteen vaccinated animals which survived the experiment, seventeen produced living calves (average duration of pregnancy 275 days) though three were infected. *Br. abortus* (infecting strain) was present in the milk of two of these three animals. A further group of forty heifers was divided into four groups of 10. Two were vaccinated with Strain 45/20 and two were kept as controls. In the 5th month of pregnancy they were infected as above with 150 million (two groups) and 1 million (two groups) organisms of Strain 544. This experiment was carried out before that detailed above and no similar fifth group of unvaccinated animals not exposed to infection was kept as a control on the possibility of extraneous infection.

When an animal aborted, samples of blood, colostrum, cotyledons (or vaginal swab) and allantoic fluid were taken from the dam, and samples of stomach contents, lung, liver, kidney and spleen from the foetus, and examined culturally and biologically. If the calving was normal the foetal samples were not obtained. Of the eighteen vaccinated animals which survived the experiment, two aborted, one calved prematurely and seven showed infection. Of the eighteen controls, five calved normally, six prematurely and seven aborted; sixteen were infected. The authors, therefore, conclude that Strain 45/20 is a poorer immunizing agent than Strain 19. They carried the experiments with 45/20 further, by injecting ten cows in early lactation with this strain and examining milk and blood samples. No evidence of *Brucella* infection was found and the blood titre did not rise. Nevertheless, on calving, examination of foetal membranes, colostrum and blood showed that five of the nine cows were infected with *Brucella*. This appeared to be a smooth strain unlike 45/20.

The authors then studied the persistence of Strain 45/20 in the tissues by injecting fourteen cattle. The animals were then killed at varying intervals from 7 to 80 days, and cultural and biological tests made from the organs and lymph glands. Five of the fourteen animals were infected, an interesting point being that three of these animals were the last three killed, while all those killed up to 38 days after inoculation were culturally negative. The majority of the strains were oxygen sensitive and did not grow in air. All the strains recovered in the whole series of experiments were examined in detail for their cultural, serological and infective properties, and the authors conclude that 'while the number of animals involved is small the evidence points to the fact that strain 45/20 after inoculation into the non-pregnant cow may in some animals persist in the tissues and subsequently undergo mutation from the rough state to a smooth virulent form'.

These results have been given in some detail, since they have been adversely criticized by McEwan(213), the originator of Strain 45/20. After giving figures of his own and reviewing the literature, to show that aerobic strains of *Br. abortus* occur naturally, and that infected animals do

not always show significant blood titres to the agglutination test, he makes the definite assertion that 'aerobiosis has been found a constant characteristic of Strain 45/20'. As the strains recovered by Edwards *et al.* were mainly CO_2-sensitive, he considers that they were field strains and that the authors placed undue reliance on the negative agglutination test and their methods of isolation. He also suggests that strains of variable characteristics isolated from the experiments, where the infecting organism was Strain 544, originated from this strain and not from the vaccine, since they were found in the group inoculated with the larger dose of 544. Another point he makes is that should Strain 45/20 mutate with relative ease then the expectation is that its vaccinal powers would be great, since it is well known that naturally infected animals have a very high resistance to reinfection. He has reported (214) his final results on the vaccination, under strictly controlled field conditions, of 114 herds. Virgin heifers were vaccinated twice and adult stock once before service. Any negative reactors at calving were revaccinated. His results are analysed in a series of tables mainly relating the abortion rate, the agglutination titre, the number of calvings and the degree of infection in the herd. He bases his conclusions on these figures and does not give any bacteriological data, since presumably these were not obtainable under field conditions. He gives figures to show that if herds are not vaccinated, then over a period of several years (5 in his case) the proportion of pregnancies per annum ending in abortion will be over 10% in 41% of the observations, between 5 and 10% in 31%, below 5% in 22% and none in 6%. Thus the disease persists and abortions occur in succeeding years, particularly during the second pregnancy. Vaccination with Strain 45/20 reduced the figures for abortions in second calf cows to 0·7% and in older animals to 0·5%. He therefore considers this a highly successful vaccine.

In view of the doubt raised by the previous workers as to its stability, this vaccine was withdrawn from issue by the Ministry of Agriculture and Fisheries, who now issue only Strain 19 vaccine, but the opposing literature has been rather fully dealt with here as there is some doubt as to which view is correct. Should McEwan be right and Strain 45/20 not mutate, then this strain would in many respects be an ideal vaccine, especially for eliminating the disease entirely. As it does not produce agglutinins it can readily be employed in any scheme utilizing a test and disposal policy—the only means at present available of freeing herds entirely from *Br. abortus* infection.

Strain 45/20 vaccine was tested in a small group of seven calves by Buddle (208) in New Zealand. After exposure to artificial infection three calved normally (one yielded *Br. abortus* in the colostrum), two aborted and one calved prematurely. One was barren. In a similar group of five which were vaccinated with Strain 19, none aborted, four calved normally (none gave *Br. abortus* in the colostrum) and one calved prematurely (*Br. abortus* present). In a field trial of Strain 19 vaccine there were twenty-two abortions in 465 first pregnancies of vaccinated heifers (an abortion rate of 5%). The previous year the abortion rate in first pregnancies had been 32%. This was repeated on a wider scale the following year, when of 10,725 first pregnancies in vaccinated animals 3% terminated in abortion compared with 22% the previous year.

Haring, Traum & Maderious (215) emphasize that Strain 19 is not by any means an ideal vaccine, particularly as it is difficult to control in preparation and storage. When in good condition it is a satisfactory vaccine, and the authors consider it could be used with advantage in animals older than 4–8 months, since they find the blood titre soon regresses.

A strong argument in favour of Strain 19 vaccine is that this vaccine has now been used on a vast and world-wide scale with remarkably few reports of mishaps. Deem & Cross (216) report on two herds, where it apparently caused abortion in cows negative to the agglutination test, and from which the organism recovered was culturally Strain 19, while Danks (217) records a case of a bull in a *Brucella*-free herd which developed a unilateral orchitis after vaccination. The testicle was removed and cultures of *Br. abortus* with the characteristics of Strain 19 were recovered. That the strain may infect the human subject is shown by Gilman (218), who records that a student who sprayed some drops of culture into his eye contracted typical brucellosis, Strain 19 being recovered from his blood.

Some reports of vaccines other than those above have appeared. Bendixen (219) records the use of a living vaccine prepared by him, and of another issued by the State serum laboratories. In

seven herds the percentage of reactors with blood titres over 1:200 fell after vaccination from 36·4 to 11·6%. As he states that 80% of the animals that show *Brucella* in their milk have blood titres over 1:200, he considers that vaccination has greatly reduced infection of the milk. In three further herds where more intensive calfhood vaccination and segregation was practised, the percentage of reactors fell after 1–2 years to about 10%, and these consisted mainly of cows with low persistent titres. These could be drafted from the herd and the herd finally cleared. He advises, however, that so long as the surrounding farms are still infected, vaccination should be continued.

These vaccines are all living cultures. Live, Sperling & Stubbs(220) have found that a dead vaccine produced by vibrating cultures of *Brucella* at 9000 cycles/sec., centrifuging, and extracting the supernatant liquid with ether (sonic vaccine) will protect guinea-pigs against infection with virulent *Br. abortus*. They also report favourably on a vaccine prepared by killing *Brucella* with ether.

Finally, a book by Huddleson(221) may be mentioned, which reviews the whole question of brucellosis in man and animals.

III. TUBERCULOSIS

A. *Bacteriology*

For general diagnostic purposes the American Committee on laboratory procedures(222) recommends shaking the sample with 2–3% NaOH and inoculating it into a simple medium consisting of equal volumes of egg yolk and 8% glycerol potato water. Van Vranken(223) prefers Corper's method of treating the material with Na_3PO_4 followed by sowing on oxalic acid egg-yolk medium. Corper & Stoner(224), in describing this method, state that a 10% concentration of the anhydrous salt is equivalent to 23% of the hydrated and that this strength is quite as effective in killing contaminants as 3% NaOH or 5% oxalic acid. It has the advantage that it does not affect the viability of *Mycobacterium tuberculosis* for 2 weeks at room temperature or 1 week at 37° C., and the authors recommend that routine specimens be treated for 2 days at room temperature or 1 day at 37° C.

Friedmann(225) reports that *Myco. tuberculosis* will grow as rapidly as any other bacteria in 12-day-old chick embryos. He treated his material with 6% H_2SO_4 and inoculated 1 ml. of the washed deposit into 25 ml. of Tyrode's solution to which he had added 0·5–1·0 ml. minced chick embryo and 300–500 units of penicillin. Good growth occurred in 3–10 days. If incubation was continued for 2–3 weeks the different types of tuberculosis could be differentiated on morphological grounds. The human type showed medusa head-like colonies, the bovine compact colonies, and the avian widely scattered loose groups of organisms. Boissevain(226) also found it possible to differentiate between human and bovine strains by growth in artificial media. He states that if Long's synthetic medium, to which 0·1% pyruvate has been added is used, bovine bacilli will grow well, whereas they are unable to grow in this medium in the absence of the pyruvate. This material does not affect the growth of human type tubercle bacilli. Solomides(227) reports that the bovine type disintegrates when grown in the presence of arachis oil, all acid fast forms disappearing in about 30 days, while Gastinel, Nevot & Hebrard(228) found that if olive oil is flooded on the surface of Lowenstein's medium the growth of both avian and bovine tubercle bacilli is inhibited. Hedvall(229) draws attention to the possibility of incorrect positive diagnosis, especially in bronchiectasis, if cultural methods alone are used. In a series of 1650 specimens, he found that sixteen gave positive cultures of acid-fast bacteria which actually proved to be saprophytes. However, while morphologically they resembled *Myco. tuberculosis*, all but one grew much more luxuriantly than this organism, and it is unlikely that they would have been accepted as such without further test by the bacteriologist examining the cultures. For biological test the guinea-pig is still the most suitable animal. Corper & Cohn(230) carried out quantitative assays of the infective dose and concluded that these animals are more susceptible than the hamster. These findings were confirmed by Steenken & Wagely(231), who found the golden hamster

resistant to doses of 30,000–60,000 organisms of a virulent human strain, doses which produced widespread tuberculosis in guinea-pigs. Even 24 hr. after infection the latter showed marked reaction to skin tests with tuberculin but the hamsters were not sensitized. Whatever animal is used, as Hauduroy, Bourier & Rosset (232) point out, the preliminary treatment of material with H_2SO_4 kills a proportion of the mycobacteria before they are inoculated into the animal. As untreated samples kill approximately 20% of the guinea-pigs used, they suggest that sulphanilamide be mixed with the injection and state that where this is done the death-rate is reduced to 6%. Schmid & Kuncaitis (233) detected open cases of tuberculosis in reacting cattle by inoculating guinea-pigs with sputum or milk (repeated samples were necessary), using 4% potassium dichromate for the preliminary treatment. They also used sulphonamide (injected in another part of the body) to aid the animals' resistance to intercurrent infection. Examined by this method, the incidence of positive sputa varied. In one area 247 out of 1682 (14·6%) were positive, while in another, 25% of 307 yielded *Myco. tuberculosis*. From 152 mixed herd milk samples two or 1·3% were positive. In Britain a similar sample of bulk milk (undesignated) was examined by Thomas (234). He reports that 0·78% of 1275 samples yielded tubercle bacilli. A wider investigation by Christoffersen, Ottosen & Thomsen (235) was used by them to compare the value of fluorescent microscopy (using auramin) with the usual Ziehl-Neelsen stain. In all they examined 1525 milk samples, 692 samples of uterine discharge, 277 of bronchial secretion and 150 lymph nodes (from pigs). On the whole, more positive samples were detected by the fluorescent method, which was particularly valuable for milk samples. Smears from discharges and lymph nodes were not so satisfactory. Cameron & Castles (236) compared the direct, NaOH, autoclave and chlorox methods of treating the material previous to microscopical examination. In all they found seventy-five positive samples, of which seventy were positive in the chlorox-treated samples, fifty-seven in the NaOH, sixty-two in the autoclave and thirty-nine in the direct method. The chlorox method was fully described by Oliver & Reusser (237). Instead of heat Muller & Chermoch (238) used Tergitol No. 7, a wetting agent, to aid the staining of acid-fast bacteria. They advocate fast green as the counterstain and consider this method particularly good for tissue sections.

Even susceptible species of animals are not all equally susceptible as individuals. Thus Diehl (239) selected fourteen lines of rabbits, some of which showed marked susceptibility and some marked resistance to bovine tuberculosis. When these rabbits were inoculated with 0·01 mg. of a virulent bovine strain, marked differences in the survival time and gross lesions were noted. In one line the only gross lesions were in the thorax, while in another the thorax showed only small reddened spots, while the skeletal glands and joints showed marked localization. These differences were inherited. Geissler (240) suggests that three factors play a part. One is an actual predisposition to the disease and the others are a specific receptivity and a specific reactivity. The actual mechanisms involved in the resistance of an individual or species are, however, still very undecided. Kayne (241) points out that the suggestion that the highly susceptible negro is highly susceptible because, not having been exposed to infection in childhood, he has no healed primary complex, is not correct. In fact he has not sufficient inherent resistance to produce such a lesion and it is therefore not there to alter the character of any reinfection. Kayne differentiates the following degrees of resistance: (*a*) not sufficient to prevent the first contact causing an extensive primary complex with rapid generalization and death; (*b*) sufficient to give a primary complex, with recovery but no acquired resistance, so that a fresh infection is liable to cause a fresh primary complex, and if under bad environmental conditions to lead to generalization and death (typified by the negro); and (*c*) sufficient to give a healed primary complex with acquired resistance. Reinfection gives a parenchymatous lesion and not a further primary complex. The young bovine would appear to fall into class (*b*), Da Conceicao (242) recording that the primary focus in 20,000 calves was in the liver in 48%, the lungs in 39% and the digestive tract in 13%. Generalization in such immature animals occurred as a miliary spread with all the organs and lymph nodes affected. Reinfection from a primary focus gives a chronic organic disease and in the older animals may cause udder tuberculosis. The miliary type of this latter is rare, 90% of cases being the lobular infiltration type. A caseous form of mastitis occasionally follows the chronic type. Stamp & Wilson (243)

survey the question of the pathogenesis of tuberculosis in the bovine and agree that haematogenous spread is of great importance, and particularly so when the glandular involvement is localized in the lung and its associated lymph glands. They showed that if tuberculosis was present in any part of the body then 4·1% of heifers, 6·3% of bullocks, 4·2% of cows and 13% of bulls showed haematogenous spread not revealed by the examination of only the lymph glands adjacent to the primary focus. They also studied the distribution of the primary organ complex. There were 189 tuberculous calves, of which 51% showed lung complexes and 20% lung and liver complexes. Only one calf showed an intestinal lesion only (0·5% were regarded as congenital tuberculosis). In heifers the lungs (22%), and the lungs and liver (13%), were again the largest groups, except that head lesions had risen from 2% in calves to 20%. This type of lesion was still more prevalent in bullocks (33%), while lung lesions remained high (26%). Lung and liver lesions dropped to 6%. This distribution was also found in bulls (head 37%, lungs 25·6%), but not in cows which showed the heifer type of distribution (lungs 36·8%, head 9·2%). In all groups except cows, primary intestinal lesions were relatively rare, especially when uncomplicated with lesions in other parts.

The authors conclude that tuberculosis in older animals is not due to the slow progression of lesions acquired during calfhood, but is a primary infection predominantly pulmonary in origin. Post-primary haematogenous dissemination also occurs. Stamp(244) also studied tuberculosis of the udder. In his first paper he gives the results of the detailed examination of twelve tuberculous udders, dividing them into four types as follows:

Type I (two cases). Acute caseating. Widespread destruction of lobular tissue, much haemorrhagic necrosis, abscess formation, occasional calcification. Microscopical examination showed rapidly progressive and caseating lesions with complete destruction of interlobular connective tissue.

Type II (two cases). Chronic diffuse granuloma: udder dry, chalky white and solid, lobules projecting above surface, no milk, supramammary glands not affected. Microscopically the interlobular connective tissue was extant but the acini were destroyed and walls of the interlobular ducts greatly thickened and their epithelium destroyed.

Type III (six cases). Chronic nodular: the cut surface appeared as a non-lactating udder with projecting nodules 2–10 mm. in diameter. These were sometimes gritty and sometimes fibrous. The supramammary glands were usually involved. Microscopically the lesions were intralobular with a peripheral zone of tuberculous granulation tissue surrounding a caseous area with central calcification. The interlobular duct walls were thickened, but the epithelium was also proliferating, and even showed papilliform projections.

Type IV (two cases). Diffuse galactophoritis without nodules: udder fibrous and pink with yellowish non-projecting lobules. Microscopically the interlobular milk ducts had thickened and occasionally keratinized walls. The lumina were filled with inflammatory exudate.

The author points out that while his Types I and II are easily diagnosed as abnormal during life, his Types III and IV (the majority) would be difficult or impossible to diagnose. He considers that the initial infection is not lymphogenous but haematogenous with primary localization in, and subsequent extension via, the milk ducts.

On this theory every case of tuberculosis of the udder is an open case, since from the earliest lesions to the final destruction of the gland the tuberculous focus is in contact with the duct system.

Steward(245) also considers that udder infection is primarily haematogenous in origin and suggests that the supramammary lymph gland is only affected after the udder has been infected for some time.

Stamp(246) has also investigated the distribution of the lesions in the lung in 100 cases of thoracic lesions. The alimentary canal, liver and head were examined, but the major attention was paid to the heart and lungs. These were either infused with 2% formol saline and immersed in formalin until fixed or frozen in salt and ice. The lungs and glands were then cut into thin slices using a ham machine and the distribution of the lesions recorded according to the major bronchial pulmonary segments. In eighty-nine of the 100 cases primary foci were recognized in the lung, while in eight

the lesions were too advanced to be recognized as primary. In these eighty-nine the lymph gland lesions were demonstrably secondary to the lung lesion. Diagrams are given showing the distribution of the lesions within the various lobes of the lung and a table relates the pathological lesion in the lung with the lesion on the regional lymph gland. He reports that 124 primary foci were seen of which seventeen were multiple. In 25% the focus was less than 1 cm. in diameter and in some was only 2 mm. In appearance they varied from an early cellular infiltration to a large encapsulated caseous calcified mass. Extension from the primary lesion was mainly bronchial and in advanced cases bronchial pneumonia resulted from ulceration of the primary lesion. The author considers that while the pathological evidence supports the view that acquired resistance resulting from the primary infection considerably alters the animal's reaction to subsequent infection, such primary foci do not usually become completely encapsulated but rather tend to bronchial dissemination and chronic pneumonia. He therefore deprecates the use of the term 'primary complex' as he considers this misleading, as the lesion in the human typified by this term is not reproduced by the cow. Stamp's conclusion that inhalation tuberculosis with primary foci in the lungs is common in the cow raises the question of how tuberculosis is present in the inhaled air. Smith, Urabec & Mason (247) tested the dust collected in dishes on the floor, air passed through cotton filters, and air passed through sterile water from rooms in a sanitorium where numerous cases of open tuberculosis were housed. In no case did they recover tuberculosis bacteria by either culture or animal inoculation. The method of spread still remains debatable, but as student nurses are often infected it would appear that ordinary hygienic precautions are not sufficient.

Climate may affect the degree of infection of any group of cattle if Rainey (248) is correct in his claim that in Fiji and Tasmania the tuberculosis figures vary according to the rainfall or rather the period of sunshine. In the wet belts of Fiji they are 20% against only 2% in the dry areas, and similar figures hold for Tasmania. McKay (249) found that while diet did not prevent the actual infection of experimental animals (pigs in his case) it did affect the rate of spread. He infected two groups of five pigs (tuberculin negative) with 1 mg. of bovine culture *per os*, and fed one on a good diet and the other on a bad. On post-mortem 16 weeks later the group on the low diet showed a more generalized and active disease, but all the animals were infected with progressive tuberculosis. Birkhaug and Schjelderup (250) also studied the resistance of experimental animals to tuberculosis. They used three groups of guinea-pigs: (1) iathergic immune, (2) allergic immune, and (3) allergic controls. They established preliminary blood levels, injected 37,000 *Myco. tuberculosis* intraperitoneally and took blood samples on the 11th, 17th, 24th, 33rd, 38th, 45th, 52nd and 58th days after infection. The most reliable guide to the progress of the disease was the leucocytic ratio followed by the monocyte percentage. The increase in circulating monocytes occurred some time before the decline in lymphocytes, but the course of the lymphoplenia is a better prognostic sign, as it is proportional to the loss in resistance. The highly iathergic immune group showed no abnormal deviations, but both the other groups showed the typical changes.

B. *Control*

Tuberculosis control varies greatly in different countries. In Scandinavia Suurballe (251) reports that successful results have been obtained in Bornholm by successive increases in the severity of the regulations as the disease became rarer. In 1937, 33% of 3818 herds were infected (in other parts of Denmark the infection rate in a representative 30% of the herds was 22%). By 1943 the price for milk not produced by herds in the eradication scheme was reduced and measures for preventing the spread by artificial insemination introduced. In 3 years the disease was eradicated (by 1946). In Switzerland legislation was introduced in 1942 (Anon. (252)) setting up an insurance scheme. This was voluntary, the Federal Government paying 50% of the expenses and 80% of the compensation. All reactors had to be marked and removed from the herd. Flückiger (253) states that in 1943 all reactors had to be slaughtered in areas where tuberculosis was not prevalent. When moderate infection occurred the reactors were segregated and disposed of gradually, while where the disease was rife the stockowner was required to remove all open cases and to maintain

the best possible hygienic conditions. In small herds no proper segregation of reactors is possible, especially as the incidence frequently reaches 50–60% (Balmer(254)). This author reports that 40% of the slaughtered reactors show only lymphatic but no organic lesions and that therefore a large-scale field experiment has been initiated to attempt to utilize such reactors as long as possible. All non-clinical reactors are removed from the herd, which is in process of being cleared. They are marked and placed in a commercial herd from which they cannot be sold and are subject to strict veterinary supervision. During 7 years 1131 animals were so transferred, of which 272 had been slaughtered and examined. Of these, 40% showed open lesions, 33% closed lymph node infection, 18% no lesions and 9% other diseases. This scheme is similar to that propounded for Britain by Savage(255), who advocates that as reactors with no clinical signs rarely give tubercle bacilli in their milk they should be kept on separate farms containing nothing but such reactors and all their milk pasteurized. Their calves should be segregated from the adults and fed on heated milk. He points out that this is, in effect, what is occurring in England, but under the worst possible conditions. A producer selling 'accredited' raw milk often does so from a herd consisting of the reacting cows from his 'attested' herd. Under Savage's scheme this would not be possible and all milk from tuberculin-positive cows would be heat-treated. Whether or not such a scheme is practicable the problem of bovine tuberculosis in children remains serious. Lethem(256), states that although in the adult 70% of human non-pulmonary tuberculosis is due to the human type, in children under 5 the vast majority of cases are due to the bovine bacillus. The effect of heat treatment in reducing the incidence of this disease can therefore be assessed by comparing the infection rates in such children in different areas corresponding to the degree of change in the amount of milk heat-treated before consumption. Thus in country districts where the milk is still not heat-treated the death-rate fell between 1921 and 1944 from 252 per million to 60 (quarter the previous rate). In small urban districts (little heat-treatment) it fell from 366 to 42 (reduction to one-ninth), in county boroughs (considerable heat-treatment) from 437 to 35 (reduction to one-twelfth), and in London (milk almost entirely heat-treated) from 136 to 6 (reduction to one-twenty-third). Lethem considers that this differential reduction is evidence of the efficiency of heat treatment in reducing the incidence of infection.

Clayson(257) states that in Scotland there are 3000 cases per annum with 400 deaths. He reports that 8–10% of milk samples contain tubercle bacilli and that 0·5–1% of cows have tuberculous udders. Milk-borne human tuberculosis is due to such animals and Faulds(258) traced six cases of tuberculous meningitis in children direct to the cows. During the early war years there was an increase in tuberculous meningitis in Wales which was investigated by Williams & Milne(259) to see if it was due to the bovine type. They concluded that their figures did not indicate any important change from the pre-war ratio of bovine to human type infections. Henschen(260) records that in Sweden 24·7% of 304 brain infections, 18·5% of bone and joint, 49% of 291 cervical, and 4·9% of 1814 cases of pulmonary tuberculosis were of bovine origin, which in forty to fifty children was traced back to a single cow. Hedvell(261) records that 3% of the pulmonary tuberculosis he typed was due to bovine infection all being in rural populations or in people infected in the country. Of sixty-seven cases of bovine tuberculosis twenty-eight died, and he emphasizes that this type of infection is clinically identical with the human type and just as fatal. Magnusson(262), commenting on this work, points out the great danger of human or bovine infection from a human carrier attending a tuberculosis-free herd and deplores the fact that there is no power to investigate the personnel in herds where bovine tuberculosis is present. Madsen(263) does not agree that bovine tuberculosis transmitted via the milk is a serious disease. He bases this conclusion on the fact that human pulmonary tuberculosis is equally common in all parts and that exposure to the bovine type of infection, though it causes a high percentage of positive tuberculin reactors among the younger population, is not followed by a comparably high percentage of tuberculous disease as diagnosed by X-rays. He qualifies this statement, however, by observing that aerogenous infection is much more dangerous than infection by ingestion, no less than half the cases in adult country dwellers in Jutland being of this type. Jensen, Lester & Tolderlund(264) also comment on the amount of pulmonary tuberculosis due to the bovine bacillus in adults living in the country. They too consider

it a rapid progressive disease. They also recorded fifteen cases of primary conjunctival tuberculosis in country patients. Griffith & Munro (265) deny that inhalation is a common method of infection of man with bovine type bacilli. They state that a searching inquiry into the environmental conditions of cases of pulmonary tuberculosis failed to show any evidence of transmission of the bovine type either from animal to man or from man to man. They examined 2769 cases in Scotland and found 160 bovine infections (5·7%), while the corresponding percentage in England, Wales and Ireland was only 1·9% (81 out of 4194). They examined all the 241 bovine cases (in only fourteen was post-mortem material available) and state that seventy gave clinical or post-mortem evidence of primary alimentary tuberculosis. In Holland previous to 1939, 9·6% of all cases of pulmonary tuberculosis and 17·2% of non-pulmonary tuberculosis in children under 15 was caused by the bovine type. In the period 1940–4, owing to bad housing, shortage of hospital services and malnutrition the incidence of tuberculosis rose considerably, but in Amsterdam, where all milk was pasteurized, the incidence of bovine tuberculosis in children fell to 1·8%. An interesting observation was a marked increase in cervical tuberculosis in the age group over 50 due to the human type (Ruys (266)).

Whatever the actual route of infection may be in certain cases there is no doubt that there is a considerable morbidity rate in man which could be reduced or eliminated by adequate control of the disease in cattle. Thus Lindau (267), reviewing the frequency of bovine tuberculosis in man in different countries, states that he found 8·9% of all infection due to the bovine type.

Francis (268) urges that in Britain eradication on an area basis with adequate compensation to the farmer should be introduced immediately, since total eradication throughout the country is impracticable. Ritchie (269) reports that in some areas progress towards eradication has been considerable. Outstanding districts at the end of 1947 were: Bute 84% of cattle in attested tuberculosis-free herds, Ayr 79%, Cardigan 72·5%, Carmarthen 64·6%, Dumfries 61·6%. In all 1,199,929 cattle in 30,436 herds were clear while a further 250,000 cattle were in tuberculin-tested herds, i.e. herds in which the milking stock was free of tuberculosis. Simms (194) gives the corresponding American figures as 65,981,099 cattle in 6,035,783 herds. In the year 1946 he states that 8,454,463 were actually tuberculin tested with 19,464 reactors, a percentage of 0·23. The effect of this work is shown not only by the reduction in reactors but also in the reduction of condemnations for tuberculosis. Thus Wight (270) comments that, while in 1917 1 in every 48 carcasses showed tuberculosis, the proportion in 1945 was only 1 in 2488. Smith (271) gives slightly different figures for carcasses condemned for tuberculosis. He states that in 1917, of 9,276,049 carcasses 40,746 were condemned (0·43%), while in 1943 of 11,559,167 only 1248 were so condemned (0·01%). His figures for the intervening years show that the drop has been steady, every successive year showing an improvement. The figures for 1944 are stated by Myers (272) to be similar (12,900,544 cattle slaughtered, 1435 or 0·011% condemned). This author states that 287,689,953 tuberculin tests have been made in the United States in the effort to eradicate tuberculosis and that, since 1917, 3,911,414 reactors have been slaughtered. The mortality and morbidity from tuberculosis in the human has also rapidly declined; in one city it has fallen from 47·3% of the children to 7·7% (tuberculin reactors); the author considers that the decline in bovine tuberculosis is the main cause of this.

Whether there is direct transmission of tuberculosis from animal to man other than by milk, there is no doubt that there is transmission from man to animal. A recent report by Tice (273) has shown how an owner can repeatedly infect a herd with bovine tuberculosis. In this case the entire herd was slaughtered several times before the source of infection was traced to bovine pulmonary tuberculosis in the owner. This type of infection is of considerable importance in Scandinavia where, as Hillermark (274) points out, the differentiation of the type of infection in a herd is essential, as different methods of control are employed according to the type of infection. Avian is distinguished by the usual comparative test and human by the Finnish 'skipping' method (the repeated intradermal injections of double doses of tuberculin, the reaction decreasing and often missing an injection). He states, however, that laboratory determinations of the type of infection is important. Jørgenson (275) comments that large farms are more often infected with avian tuberculosis than

small ones (18·6% compared with 2–3%), but that the percentage infection rate within the farm is greater in small than in large farms. He found that 90% of the avian infection occurred in farms previously completely free of any type of tuberculosis and that it tended to disappear after 4–6 years. Another problem in Scandinavia reported by Hermansson (276) is congenital tuberculosis. Wherever a calf is found on meat inspection to be tuberculous, a clinical and bacteriological examination is carried out on the dam. If this reveals tuberculosis the animal is slaughtered. As extremely few calves are slaughtered, he suggests that the vaginal exudate and foetal membranes in every case of abortion should be examined for tuberculosis and that all calves should be tuberculin tested. As an instance of the damage an infected cow can cause he gives a case where tubercle bacilli were found in the vaginal discharge of a cow and, though she was immediately slaughtered, nearly the whole herd of 100 animals reacted 3 months later. Uterine discharges can be very infective, two out of twenty-five guinea-pigs dying when injected with 0·00001 mg. of exudate. Fomite transmission is also possible, since he records a case of a veterinary surgeon attending an infected cow and then castrating some pigs on a neighbouring farm. The latter developed and spread tuberculosis. Berger (277) in Britain found tuberculosis in twelve out of 128 cases (9%) of uterine discharges and reports that many of the affected cows showed no obvious signs of tuberculosis. (Such animals, though cases of open tuberculosis, cannot be slaughtered, nor can compensation be paid under the tuberculosis order.) He emphasizes the dangers of primary uterine tuberculosis due to fomite transmission during assisted calvings or placental removals.

The ease with which very small numbers of bacilli can infect an animal is shown by Riordan's results (278). This author produced primary rectal tuberculosis in twenty-nine out of 212 monkeys by using contaminated thermometers. Another case involving cows is that related by MacFarlane, Garside, Watts & Stamp (279). An attested herd of eighty-seven animals had shown no reactors for 5 years and then at one test thirty-two of the thirty-three milking animals reacted and the only non-reactor showed *Myco. tuberculosis* in her milk. The bull, three out of seventeen yearling heifers and thirteen out of fourteen calves also reacted, the only group of non-reactors being twenty-two bulling heifers out at grass. The history was that some 7 months previous to the test twenty-one quarters of sixteen dry cows had been irrigated twice with acriflavine using the same apparatus for all the cows, and injecting the dye under positive pressure. The irrigated quarters slowly became enlarged and indurated and further treatment did not improve the condition. Ten of the cows were slaughtered and passed the meat inspectors. When the tuberculin test revealed that the remaining cows were tuberculous, the milk was examined and all six remaining of the irrigated cows were found to be shedding *Myco. tuberculosis* in their milk. Detailed post-mortem examinations showed that all had primary tuberculosis of the udder in the irrigated quarters. Other tuberculous lesions were secondary. Only two non-irrigated cows were available for post-mortem and neither of these showed udder tuberculosis, both being cases of early tuberculous bronchial pneumonia. Since the whole herd was negative to the tuberculin test before the irrigation, and none of the animals slaughtered previous to the batch examined was condemned for carcass tuberculosis, and since none of the calves or stirks fed with milk or in contact with the herd previous to the irrigation showed tuberculosis, while every animal in contact after the irrigation was tuberculous, the authors conclude that there is irrefutable proof that the infection was introduced by the irrigation.

Another cause of tuberculosis without direct contact is the presence of tubercle bacilli on pasture. Christiansen (280) studied this by running open cases on a field for 10 days. He then removed the animals and replaced them by non-infected heifers after varying intervals. He found that no infection occurred later than 7 days after the cows were removed, though a water pond artificially contaminated remained infective for 18 days. Manure was found not to be infective if spread on the field.

The incidence, pathogenesis, detection, vaccination and control of bovine tuberculosis has been recently discussed in book form by Francis (281).

C. *Tuberculin*

Green(282) has recently reviewed the production of tuberculin at Weybridge, the central laboratory for the issue of tuberculin in Britain. This is now all purified protein derivative (P.P.D.) and the author points out that this laboratory produces more of this product than any other in the world (approximately 10 million doses per annum). It is prepared from the human strains used by the American Bureau of Animal Industry (D.T., P.N. and C.). These are surface-sown on a synthetic medium containing 0·18% $K_2HPO_4.3H_2O$, 0·09% sodium citrate, 0·15% $MgSO_4$ (crystals), 0·03% ferric citrate, 1·4% asparagine, 1·0% glucose and 10% glycerine in distilled water. 2 g. of bacterial dry weight is produced after 10 weeks' incubation at 37° C. when the culture is steamed to kill the organism. It is then filtered through paper pulp, the protein precipitated with 40% trichloroacetic acid, washed in dilute trichloroacetic acid, centrifuged and resuspended in slightly buffered water to remove the acid. It is then recentrifuged, suspended in M/15 Na_2HPO_4 and diluted to issue strength. M/15 NaOH, glycerol, phenol and NaCl are then added to give a pH of 7 and a final concentration of 10% glycerol, 0·5% phenol and 0·5% NaCl. This product is spun in a Sharples super centrifuge to remove protein of high molecular weight, diluted to standard (biologically assayed) and filtered through sterilizing pads into phials. Avian tuberculin is prepared similarly, but is more concentrated as it is less potent. P.P.D. has also been prepared from bovine strains, but these yield less than the human type (about 1/5). However, a bovine strain has been found which will grow well under these conditions and produce equivalent amounts of tuberculin. Green gives a chart showing the cross-reaction of six different types of P.P.D. when tested against the corresponding 6 infections in guinea-pigs. This shows that P.P.D. from human, bovine and B.C.G. organisms is practically identical, but that avian is twenty to forty times, johnin ten times and phlei 150 times weaker. The allergic reaction is produced by a primary protein unit of molecular weight about 8000. These units may aggregate into larger molecules and still give the reaction, but no smaller molecule has been found effective. P.P.D. of small molecular weight is of particular value as it does not sensitize the skin of a non-reactor so that repeated tests can be performed without local allergy being produced. P.P.D. of larger molecular size does sensitize the skin. While the Weybridge P.P.D. is prepared from the human strains of *Myco. tuberculosis* used in America it is rather less concentrated than that used in that country. It is, however, used at double the dose (0·1 ml. as against 0·05 in America). Paterson(283) has now produced a potent P.P.D. tuberculin from a bovine strain. This strain, Weybridge AN5, is a eugonic variety and grows well in 10% glycerol medium giving a good yield of tuberculo-protein. He considers that the type of growth affects the yield of P.P.D., an expanded thick fluffy growth yielding better than a tight granular growth, but that the most important factor affecting the yield of a tuberculinogenic strain is the maintenance of an optimum pH for the autolytic degeneration of the bacterial cells, since he agrees with Corper & Cohn(284) that tuberculin is the end-product of such degeneration. (These authors obtained a good tuberculin from a 4-week growth autolysed in toluol for 7 days at 37° C. The pH had to be 7–8.) Bovine tuberculin has also been successfully produced by Schaefer(285).

At present by far the major portion of all tuberculin produced is from human strains, both America and Britain using this for their official tests. The latter country has recently (1947) changed from the double intradermal test to the single (Anon.(286)). This was done on the results of an experiment in which 7247 animals in attested herds with poor histories and 2244 in infected herds were tested. The double intradermal test was carried out on one side of the neck and the single on the other. The double test was read at 48 and 72 hr. and the single test at 72 hr. only. Where the tests differed in their results the animals were slaughtered and examined post-mortem. There was 99·12% agreement between the two tests in the 7247 cattle tested in lightly infected herds and 96·57% in the 2244 cattle in heavily infected herds. The total discrepancies were fifty in the former group and sixty in the latter and were mainly due to the non-diagnosis of reactors in heavily infected herds. The authors consider therefore that this is of little practical importance and the single test has been introduced. The reaction is read at the 72nd hr. and a 3 mm. increase is regarded as positive.

Even the relatively pure P.P.D. can give false reactions when injected into human beings or animals, since Kreis & Renault(287) report that marked reactions (e.g. congested and papular) can be produced in susceptible subjects by the injection of distilled water alone and they urge caution in interpreting positive reactions unless control injections are made. Andres(288) also emphasizes that local and general reactions are of even greater importance than the mere increase in thickness of the skin fold. Dalling(289), however, by inference, suggests that with the increase of purity due to the issue of P.P.D., the skin thickness is more important than the character of the reaction. Zeissig(290) used P.P.D. instead of the standard U.S. tuberculin and examined all reactors post-mortem. He found that the larger the reactor group the greater the probability of tuberculosis. In herds with only 1 reactor 77·7 % were 'No visible lesion' reactors, while in those with sixteen reactors only 25 % were in this group. He continued this in a further article(291) when he retested 408 reactors. If more than five reacted in a herd, tuberculosis was present, but if less than this number then any doubtful macroscopic lesions post-mortem were examined histologically. Of the animals, 26 % were tuberculous, 22 % had skin lesions and 52 % had 'no visible lesions'. The re-test reaction was usually less severe in the skin lesion and no visible lesion groups, though twenty, including three open cases, of the 106 tuberculous animals failed to react. This failure to react to tuberculin on re-test has been examined by Kerr, Lamont & McGirr(292). They found that parturition caused a great reduction in the sensitivity to tuberculin in a group of twenty-eight cows. Pre-calving tests had shown twenty reactors, three non-specific reactors and five non-reactors. Tests 1–14 days after calving showed that of the twenty positive animals seven did not react, five gave non-specific reactions and the remaining eight showed a considerable drop in their degree of reaction. The authors suggest that a possible explanation is that there is a mobilization of the fixed cell antibodies into the general circulation for abstraction in the colostrum. Their experiments on the passive transference of tuberculin sensitivity to calves via colostrum lead them to conclude that: (*a*) the colostrum of mammalian sensitized cows appears to possess a quantity of specific antibody substance which is not present in normal animals; (*b*) this antibody may be absorbed by the newborn calf; (*c*) these calves sometimes develop a skin sensitivity which may persist for 6 weeks. They carried their experiments on desensitization further and were able to desensitize naturally infected positive reactors by the previous injection of 10–100 mg. of P.P.D. (Weybridge). They conclude that while the mechanism of desensitization is obscure it is probable that the desensitizing procedure either destroys or neutralizes the sensitizing antibody,or, leaving this intact, creates conditions that interfere with the reactions between the antigen and antibody. They do not think that there is any alteration in the susceptibility of the tissues to the antigen-antibody complex. This desensitization does not only not occur in the area inoculated with the usual intracutaneous tuberculin dose but the skin becomes hypersensitive and this is used by the authors as the basis of their 'Stormont' test. This consists of the usual injection into the neck of 0·1 ml. P.P.D., followed 7 days later by a second injection of 0·1 ml. into the same site. Readings are accurately taken 24 hr. later when a rise of 5 mm. or more in thickness is taken as positive and 4·9 or less as negative. No doubtful class is included and no account is taken of any factor other than the increase in skin measurement. Using this technique in conjunction with the single intradermal test the authors tested 300 animals, having their results statistically analysed by Priestley(293). His conclusions were:

(1) In autopsy-negative animals there is no difference between the two tests, the maximum increases being similar.

(2) In autopsy-positive animals the single intradermal test gives a mean maximal increase of 6·8 mm. with mammalian tuberculin and significantly less (2·5 mm.) with avian.

(3) Using avian tuberculin the 'Stormont' test does not distinguish between infected and non-infected animals.

(4) Using mammalian tuberculin the 'Stormont' test gives a significantly greater increase in positive (10·7 mm.) than in negative (2·0 mm.) animals.

(5) Using mammalian tuberculin in autopsy-positive animals the 'Stormont' test gives a higher mean maximum increase (10·7) than the single intradermal (6·8).

(6) The 'Stormont' test detected autopsy-positive animals from autopsy-negative animals with an error of only 1·8%, as compared with an error of 18·9% with the single intradermal.

(The actual figures, assuming the autopsy results to be correct, were: correct positives—single intradermal 72, 'Stormont' 90; false positives—single intradermal 42, 'Stormont' 3; correct negatives—single intradermal 166, 'Stormont' 205; false negatives—single intradermal 17, 'Stormont' 2.) While they therefore advocate the use of the 'Stormont' test they draw attention to a possible danger in its use in that it gives a strong positive reaction in animals with 'skin tuberculosis'.

Two other modifications of the tuberculin test have been suggested by Chelle, Ukrainczyk-Laborie & Laborie(294) and by Zeissig(295). The former authors injected doubtful reactors intravenously with 4 ml. of subcutaneous tuberculin and found that in positive cases the temperature rose in 1–3 hr., reached a maximum at 9–12 hr. and returned to normal after 18 hr. In ten doubtful cases six gave positive intravenous reactions. Zeissig injected subcutaneously 3–4 ml. of intradermal tuberculin and took hourly temperature readings for 14 hr. He claims this detects the non-reactor spreader in a herd. Arthur(296) attempted to reduce the number of doubtful readings by including a differential leucocyte count. He states that there are increases in the total leucocyte counts and a narrowing of the lymphocytic-neutrophilic ratio in reacting cows but that no change occurs in non-reactors. The degree of neutrophilia was not proportional to the increase in skin measurement and occurred later than the maximum skin reaction. It was not possible to differentiate between reactors to tuberculosis and non-specific reactors such as skin tuberculosis cases. Beamer(297), in a similar study, considers this reaction is non-specific, since he found that the changes occurred in fourteen non-tuberculous non-reactors. Of these four were studied in detail post-mortem. Two showed *Corynebacterium pyogenes* infection, one a staphylococcal tendovaginitis and the other a granulomatous skin lesion.

There are a number of conditions other than these which affect the tuberculin test. Canham(298), in a general review of the tuberculin test, draws attention not only to *Br. abortus* infections and late pregnancy, but also to the effect of infection with the non-pathogenic Mycobacteria such as *Myco. butyricum* and *Myco. phlei*. These when injected into cattle not only gave permanent lesions indistinguishable from tuberculosis from which they could be recovered up to 13 months after injection, but also sensitized the animals to tuberculin, a sensitivity which remained even after surgical removal of the lesion itself. Brown(299) includes *Myco. johnei*, *Actinobacillus lignieresi* and parasitic worm larvae in his list of factors affecting the test. Perhaps the most important of the diseases affecting the tuberculin test is skin tuberculosis. Whether or not this is due to an acid-fast mycobacterium is still not certain, but the evidence that this is so is reasonably strong. Our knowledge of this disease, its aetiology, diagnosis, effect on the tuberculin test and differentiation from demodectic mange and nodular necrosis of cattle has been fully described in a pamphlet issued by the National Veterinary Medical Association of Great Britain and Ireland(300). (No references are given.) It will not therefore be further discussed here.

The group giving no-visible lesions which continues to be a source of much difficulty in areas of very low tuberculosis incidence is not connected with avian tuberculosis, since Fidler(301) found that on sixty-one farms where he tested 10,415 birds the incidence of the no-lesion reactor in the cattle bore no relationship to the incidence of tuberculosis in the poultry. He states that the percentage of 'no-visible-lesion' reactors per 100 reactors increased from 12% in 1930 to 32% in 1945. This, however, was not due to any increase in the total number of such reactors but to the fact that the reduction in the incidence of 'no-visible-lesion' reactors was not so great as the reduction in the total number of reactors. In this period the total reactors per 10,000 tested fell from 170 to 24 (decrease of 86%), while the similar figures for 'no-visible-lesion' reactors were 20 and 7 respectively (65% reduction), thus there was a relative increase in 'no-visible-lesion' cases.

D. *Vaccination*

There have been several types of vaccine suggested for use against tuberculosis. Thus Gräub, Zschokke & Saxer(302) found that repeated subcutaneous injections of an attenuated strain of *Myco. tuberculosis* protected cattle against subsequent exposure to natural tuberculosis, while Portela(303) and Olson, Habel & Piggott(304) used killed vaccines. The former suspended killed bacilli from a virulent strain in liquid paraffin and incubated it for 5 days. He then centrifuged the suspension and used the supernatant fluid as a vaccine. Although it was bacteria-free it produced lesions and sensitized laboratory animals equally as well as live bacilli or aqueous suspensions of dead bacilli. He used this as a method of standardizing tuberculin, since it produced a rapid uniform allergy in all the animals which are non-infective and could therefore be safely handled. The latter authors found that a vaccine prepared from cells killed by ultra-violet light was more effective against the original homologous strain than either a similar heat-killed vaccine or B.C.G. A vaccine which has been more widely investigated is that prepared from suspensions of the vole strain of *Myco. tuberculosis*. Brooke & Day(305) divided a number of guinea-pigs into four groups each containing approximately forty animals. The first group received two doses of 4 mg. of living vole bacilli, the second a heat-killed vaccine, the third 10 mg. of B.C.G. vaccine and the fourth served as a control group. Seven weeks later all received 0·000001 mg. virulent human type bacilli. After 9 months 78% of the controls showed generalized tuberculosis as against 35% in the heated vaccine group and 18% in the vole bacillus and B.C.G. groups. After a further 5 months 50% of the B.C.G. group and 29% of the vole group showed gross evidence of tuberculosis. They therefore consider that the vole bacillus vaccine is at least as good as B.C.G., especially as they could find no progressive lesions due to this organism unless very large doses were given. It would appear, however, that the vole bacillus is slightly more pathogenic than B.C.G.; Corper & Cohn(306), for example, consider that it multiplies in the tissues and produces lesions which often include the internal organs. They suggest that since the immunity it induces is about equal to that produced by the attenuated mammalian strains the latter should be used in preference. Birkhaug(307) also states that the vole bacillus has a smaller margin of safety than the mammalian types and that the skin lesions produced by intracutaneous inoculation are more vigorous and persistent than those produced by B.C.G. He prefers the multipuncture method of inoculation rather than the simple intracutaneous method, since he found in guinea-pigs that though no absolute immunity to artificial infection was produced by either type the degree of protection was markedly higher with the multipuncture type. Hilleboe(308) states that the intracutaneous method is to be preferred and recommends that all vaccine be prepared at a single central laboratory. He reports that B.C.G. vaccine definitely confers an increased resistance to tuberculosis for a limited period and that no progressive disease has been reported as the result of B.C.G. injections (this is the report of a conference which has been published(309)). Birkhaug(310) also considers that B.C.G. is harmless and that it produces a relatively high degree of protection which is almost complete against post-primary extensions and considerable against secondary tuberculosis. Hansen(311) states that the immunity produced by vaccination may be broken down by super infection with virulent bacilli, but he thinks the post-vaccinal allergic state probably lasts 4 years or more. If no allergy is stimulated the subject should be revaccinated, since immunity is associated with allergy. He cites a case where in a group of tuberculin-negative non-vaccinated nurses the morbidity was 17·1%, while in a vaccinated tuberculin-negative group it was 10·8% and in a vaccinated tuberculin-positive group 0·9%. Complications such as abscesses or sinus formation may occur after vaccination, but there is no evidence that this procedure reactivates quiescent tuberculosis. Ferguson(312) also notes the value of B.C.G. in reducing the tuberculosis morbidity in nurses (in ordinary hospitals it reduced the morbidity rate by 75% and in sanitoria by 80%). Aronson & Palmer(313) vaccinated 1550 American Indians and kept 1457 controls. The intracutaneous route was used and records were kept for 6 years. The total cases were 40 and 185 respectively with 4 and 28 deaths. In terms of cases per 1000 person-years the controls gave 24·3 compared with the vaccinated group, which gave 4·7. Direct experimental

evidence of the value of B.C.G. in the human body was produced by Turk (314), who vaccinated two infants and artificially infected these two and one unvaccinated infant. The two vaccinated did not show tuberculosis but the unvaccinated developed the disease.

Considerable numbers of children or young adolescents among the general population have been vaccinated. Hansen (311) says that in 1942, 6000 were vaccinated in Norway (usually tuberculin negative persons known to be exposed to infection). Since 1940, 100,000 people have been vaccinated in Norway, 58,000 conscripts and 250,000 civilians in Sweden and 150,000 people in Denmark (Birkhaug (310)). Wallgren (315) has reviewed the position which has existed in Sweden during the 20 years vaccination has been practised, stating that the number vaccinated reached 57,000 during 1943. In Moscow and Leningrad the number of vaccinated infants has also increased considerably (Klebanov (316)). Between 1933 and 1940 they increased from 3000 to 103,000 in Moscow and from 5000 to 48,500 in Leningrad. The infant mortality during the same period fell by 40%. Records were kept during the first 2 years of life of a group of 56,951 vaccinated and 17,469 unvaccinated infants. The mortality figures per 10,000 were: first 6 months—vaccinated 4·4, unvaccinated 5·7; second 6 months—vaccinated 8·3, unvaccinated 7·0; second year—vaccinated 10·7, unvaccinated 20·8. He considers that close contact gives a high mortality despite vaccination, especially if the contact occurs before immunity can develop. In South America De Assis (317) gives the vaccine *per os* to the newborn in tuberculous households and states that approximately half the children born are vaccinated. He found that good results depended on giving large doses of freshly prepared vaccine in a slightly alkaline fluid administered fasting. Blanch, Blanch & Lieutier (318) in Uruguay have abandoned the oral administration in favour of the subcutaneous, intracutaneous, and multipuncture methods but still find it of little value in preventing infection and death in infants exposed to massive infections in tuberculous households. They vaccinated 103,000 babies and found that the mortality rate in this group of 7% compared unfavourably with 5·2% in the unvaccinated. (From their figures it appears that the incidence rate is high and that the population at risk has a high mortality rate.) They advocate the removal of the infant from the infected household. A much smaller death-rate is reported by Rosenthal, Bladh & Leslie (319), who report three cases with one death in 1204 vaccinated children and one case with no deaths in ninety-eight infants. Similar figures for unvaccinated children and infants were 1213 children with twenty-three cases and four deaths, and sixty-three infants with four cases and three deaths. In 1946 Levine & Sackett (320) published a critical review of B.C.G. vaccination, in which they point out that over 2,000,000 children have been vaccinated and that on the whole the reports are very optimistic. They consider, however, that the controlling of the experiments has been inadequate, and cite an experiment in New York. In this the history was recorded of 1011 vaccinated and 1073 unvaccinated children from tuberculous homes. Before strict alternation of cases the control group showed four times the mortality of the vaccinated group (3·38–0·68%) but after this was introduced the figures were practically identical (1·5 and 1·41% respectively). They discuss the effect of parental co-operation, economic condition, racial distribution, exposure, lost cases and number of autopsies and point out that to be of value as a public health measure vaccination must be capable of reducing the mortality in children vaccinated in their homes and exposed to a tuberculous environment. This they consider B.C.G. vaccination cannot do and they therefore prefer to move the tuberculous subject from the home.

B.C.G. vaccination is being employed in a field trial in this country in an attempt to eradicate tuberculosis from cattle in 100 farms where there was a high incidence of the disease in the older animals and a low incidence in the young stock (Dalling (321)). Every 6 months the young stock are injected with B.C.G. and as they come to production they are used to replace older infected animals. When the herd is composed entirely of vaccinated animals vaccination is stopped. It has been found that the tuberculin test in cattle becomes negative in from 9 to 12 months after the cessation of vaccination. This experiment is not yet completed. In Russia Kokenrichev (322) reports that B.C.G. immunity in calves is not sufficient to enable them to withstand mixing with infected stock. Of 556 exposed to natural infection 132 were histologically examined post-mortem. In thirty, lesions due to B.C.G. were found, in ten, mixed B.C.G. and virulent tubercle infections and in sixty-nine virulent tuberculous lesions.

E. *Treatment*

Practically every fresh antibiotic or synthetic chemical therapeutic agent has been investigated for its value in the treatment of tuberculosis and a review of the position in 1946 has been given by Hart(323). It is not possible here even to list the large number of synthetic drugs mentioned, but the consensus of opinion is that chemotherapy has little place in advanced secondary tuberculosis where cavitation or abscess formation has occurred, but may be of value in early acute exudative types of the disease. The same applies to the antibiotic group. Penicillin does not affect the end result of the disease, and the most recent reports on streptomycin are also discouraging, though its low toxicity, and *in vitro* activity suggests that it may be of greater value than any other drug. In man its main value is as an adjunct to the more conservative lines of treatment (hygienic, nutritional, etc.) especially in cases of early or haematogenous tuberculosis (Anon.(324)). Corper & Cohn(325) consider that it is difficult to know when to use streptomycin therapy, since its action is bacteriostatic, and it is therefore only of use when the organisms are multiplying. As this phase is irregular and unpredictable in tuberculosis the value of the antibiotic is limited, especially as repeated parenteral injection is necessary to maintain an effective level in the blood. Youmans & Karlson(326) point out that *Myco. tuberculosis* acquires a considerable degree of resistance against streptomycin; cultures recovered after some months of treatment being several thousand times more resistant than normal cultures. They state that the bovine bacillus is as susceptible as the human, but it seems extremely unlikely that the drug treatment of bovine tuberculosis will ever be a practical proposition.

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